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groups and which would be of use in the estimation of radiation prognosis

Mottram (1913), Holthusen (1921), Regaud (1923), Strangeways and Hopwood (1926) and Vintemberger (1928) have described various stages of mitosis as being the most susceptible to irradiation. All are agreed, however, that mitosis is the most and the resting phase the least vulnerable to its action. Dustin (1930) says that the most vulnerable cells are those in which the chromatin is "en voie de condensation soit chromosomiale (caryocynèse), soit prépycnotique". Maishak and Bollman (1936) have suggested that the amount of irradiation absorbed will be directly proportional to the amount of chromatin in the nucleus. Scott (1930) has shown that the nuclear chromatin corresponds to the inorganic material remaining after incineration, and Scott and Horning (1932) report that the inorganic content of malignant breast cells is higher than that of benign.

With the well established observation that malignant nuclei are more hæmatoxyphil than normal nuclei there is enough evidence to indicate that both the staining properties and the inorganic ash content of malignant nuclei may afford an index to their radiosensitivity. The degree of hæmatoxyphil staining of a section varies with the nature and maturity of the stain used, and from technician to technician, and is therefore of little value in the estimation of comparative radiosensitivity. Accordingly attention was turned to the inorganic nuclear constituents as demonstrated by micro-incineration.

Cathie and Davson (1937) have published recently the findings after micro-incineration in a small preliminary series of 19 cases of basal cell and squamous cell carcinoma of which the radiation lethal dosage was accurately known. They found that there was a difference in the amount of ash left, the basal cell growths always showing a higher ash content than the squamous cancer. They also found that the distribution of ash in the individual nuclei tended to be different. In the basal cells the ash was scattered diffusely throughout the nucleus as a smear, in the squamous cells it was concentrated at the periphery of the nucleus, producing a ring appearance. The basal cell tumours all had lower lethal dosages than the epitheliomas.

This work has been repeated and extended on different types of sensitive and resistant tumours and the ash content has been correlated with radiosensitivity.

Material and methods

The material used consisted of the routine biopsies coming to the laboratory. In addition, to make the rarer growth groups more comprehensive, old blocks of such tumours as lympho-epithelioma and melanoma were re-examined.

Borak's conclusions concerning the relative sensitivity of parent and malignant tissue and explains how a subcutaneous growth may be killed without delivering a skin lethal dose

Figs 7 and 8 show normal and malignant columnar cells from different parts of the same section of a rectal carcinoma. The malignant cells leave a higher ash than the normal, again supporting Borak's statements

3 Various other tumours of known radiosensitivity were incinerated and examined. In the case of the histologically less clear-cut types of growth an insuperable difficulty was encountered in the shape of lymphocytic infiltration. Lymphocytes, polymorphs, red blood corpuscles and apparently fibroblasts all have a high ash content, and in a diffusely infiltrating or infiltrated growth such as a lympho-epithelioma it is impossible to tell from the incinerated section the type of cell under observation consequently it was found impossible to estimate either total ash or nuclear distribution. In these cases, of course, reference to the control stained section gave no help

Thymomas, seminomas and lymphosarcomas, at one end of the scale, had a very high ash content and the nuclei were all of the smear type. The ash of the thymomas was considerably greater than that of the lymphosarcomas, which in its turn was greater than that of the basal cell carcinomas. At the resistant end of the scale, the spindle cell sarcomas showed about the same amount of ash as the squamous carcinomas. It was situated in the nuclei, chiefly as the ring type, with an occasional smear. The ash of the melanomata varied constantly with the hæmatoxylin staining of the nuclei, and while some were almost ash-free others had a high content. It has been found impossible to pick out a group of melanomata of less resistance than the average, and the variation in their ash content provides no clue to their relative radiosensitivity

4 The histological variants of epithelial tumours showed little in the way of constant ash differences to account for their variations in sensitivity. The nuclei of the spindle cell epitheliomas contained as much ash as the normal squamous type, possibly even more, while the other squamous variants showed no appreciable differences

The tricho-epitheliomas contained remarkably little ash and the nuclei appeared as extremely small punctate areas in a wide ashless background. Transitional cell growths from cervix, skin and oesophagus had an ash content roughly midway between that of basal cell and squamous cancer, and in nuclear type the ash was usually arranged in faint smears

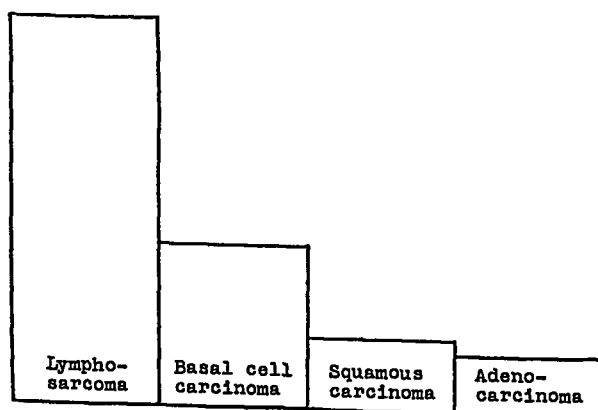
The columnar cell carcinomas of the rectum left an ash picture rather difficult of interpretation. The total ash appeared to be somewhat higher than that of the epitheliomas, but as far as could

Ten readings were taken at regular intervals across the selected area, and their mean calculated. In this way comparable figures were obtained for different types of tumour (table)

TABLE
*Photometer readings given by incinerated sections of 28
representative tumours*

	Lymphosarcoma	Basal cell carcinoma	Squamous carcinoma	Adenocarcinoma
1	6.8	2.2	0.9	0.6
2	6.1	2.4	1.1	0.9
3	7.1	1.9	1.9	1.2
4	6.1	3.3	1.3	2.2
5	6.8	2.3	0.8	0.8
6	6.4	2.7	1.4	1.0
7	5.4	1.8	1.1	
8		2.6		
Mean	6.4	2.4	1.2	1.1

The differences in the amounts of ash in these various types of tumour are more easily appreciated from the accompanying graph.



Graph showing the average amounts of ash in four types of tumour

The total number of cases quoted is small, but is representative of a much larger series examined. The figures are possibly not strictly accurate, because of the experimental error consequent upon the inability to observe the measured fields directly. This error has been eliminated as far as possible by uniform technique and selection of suitable fields for measurement.

As a comment on the atypical readings in the above series it may be worth stating that the basal cell carcinoma giving a reading of 1.9 was, although histologically typical, very resistant. The columnar cell carcinomas were all resistant with the exception of

than the resting cell. Whether this is a false impression produced by bunching of the chromatin it is impossible to say, but it may be that mitotic vulnerability is due to the bunching, which enables more quanta of irradiation to be absorbed.

Radiotherapeutic controversy is centred at the present time around the relative merits of the theories of biological variation and quantum hit. Briefly, the biological variation theory postulates that since the amount of irradiation received by each cell in a growth is the same per unit area the cells must show an individual variation in their capacity for being damaged or killed by the irradiation. The quantum hit theory assumes that all the cells have an equal capacity for being killed by the irradiation but that the amount they receive from cell to cell varies. A more precise variation of this theory is that there is some especially vulnerable spot in the cell, possibly the centriole, upon which a direct hit must be registered with one or more quanta.

Considering first the quantum hit theory, it is difficult to imagine an area as large as a nucleus not receiving a homogeneous dosage of irradiation, and to conceive of the element of chance when dealing with such a minute thing as a quantum of radiant energy it is necessary to postulate an exceedingly small vulnerable spot in the cell. Various writers have suggested that this vulnerable spot is the centriole, the nucleolus and the Golgi apparatus. The theory, which has been associated with the names of Crowther (1926), Holweck (1929), Lacassagne (1929), Curie (1929) and Mayneord (1934), is founded chiefly on the similarity between probability curves and curves of radiation mortality. It must be pointed out that the theory has been invented to fit the resemblance of these curves, and has no foundation of experimental evidence.

As it stands at the moment the theory of individual variation is equally unsatisfactory. That cells vary in many ways is a known fact, as is also the impossibility of producing a homogeneous population experimentally. The same type of cell varies in size, shape and staining properties as well as in radiosensitivity, and to say that cells vary in their radiosensitivity because they vary biologically seems tantamount to saying that cells vary because they vary.

In the present investigation the working hypothesis was postulated that the difference in radiosensitivity of various types of cell was due to the presence of some substance, varying in amount, upon which the applied radiations could act. The question as to whether the death of the cell was produced as a result of the direct radiation or by some mechanism of backscatter or secondary irradiation was not within the scope of the postulate. It is felt that this hypothesis is substantiated in fact by the finding of the variation with radiosensitivity of the nuclear inorganic ash.

writer suggests that the inorganic nuclear content is the variant upon which relative radiosensitivity depends

Summary

Work is described to show that the inorganic ash content of a malignant tumour, as demonstrated by the technique of micro-incineration, affords an indication of its degree of radiosensitivity

Current theories of radiosensitivity are briefly discussed, the biological variation theory is supported, and it is concluded that the variant in question is the amount of inorganic material in the nucleus

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Morphology

The morphology was studied in preparations from cultures from single colonies on plain agar slopes cultivated at 37° C and at room temperature (17-25° C). The morphology at 30° C. does not differ appreciably from that at 37° C.

Listerella. Rods about 0.5 μ in width, with rounded ends, arranged singly, in packets, in groups or in chains. No capsules and no spores. *Smooth form*. After 7-12 hours' incubation at 37° C, rods from 0.5 to 2.4 μ predominate with occasional longer forms. At 24 hours the culture consists almost entirely of coccobacillary forms, 0.5-1.0 μ (fig 1). At 48 hours the coccil forms are slightly more predominant. No change occurs after this. In cultures incubated at room temperature the organisms divide into small forms rather more slowly, a 24 or 48 hour culture resembling a 12-hour culture at 37° C, and even older cultures show bacilli rather than coccil forms. *Rough form*. Early cultures (7-12 hours) at 37° C consist almost entirely of long filaments averaging about 60 μ . Some of these filaments can be seen to be dividing into chains of bacilli. At 24 hours, long rods from 4.5 to 8.0 μ and filaments from 10 to 30 μ predominate, a few short rods from 1.5 to 3.0 μ are also present (fig 2). At 48 hours, rods from 1.0 to 5.0 μ predominate, short filaments from 10 to 12 μ are present in moderate numbers and there are a few coccil forms. No further breakdown takes place after 48 hours. Cultures at room temperature consist of enormous filaments with little or no tendency to split up into bacilli (fig 3). *Intermediate form*. Fig 53 XXIII shows a morphology intermediate between these two after 24 hours' incubation but at 48 hours the short filaments and long rods almost entirely split up into coccobacillary forms resembling the smooth type. Fig 6 shows the filaments dividing into rods.

Erysipelothrix. Extremely slender rods from 0.3 to 0.4 μ in width, arranged singly or in packets, groups or chains. No capsule and no spores. *Smooth form*. 12- to 24-hour cultures consist predominantly of minute rods 0.8-2.0 μ in length (fig 5), with occasional filaments. In older cultures there are rather more filaments. Room temperature cultures show little difference. *Rough form*. 7- to 12-hour cultures show organisms varying from 1.5 to 11 μ . At 24 hours, filaments from 10 to 30 μ predominate, while rods (0.8-5.0 μ) and short filaments (10 μ) are present in about equal numbers. Older cultures show little change. Cultures at room temperature show enormous filaments which fail to split up into rods (fig 7). *Intermediate form*. In 7- to 12-hour cultures at 37° C, organisms vary from 1.5 to 10 μ . At 24 hours filaments from 15 to 25 μ predominate, with some rods 1.5-6.0 μ (fig 4). At 48 hours organisms are almost entirely filamentous, averaging about 30 μ . At room temperature the organisms vary from rods of 2 to 5 μ to short filaments of 10 to 11 μ .

Staining. All strains of *Listerella* and *Erysipelothrix* show similar staining reactions. They are Gram-positive, although fairly easily decolourised. Staining is sometimes irregular but no true granules have been seen with metachromatic stains in plain agar or inspissated serum cultures.

Motility. *Erysipelothrix* strains are non-motile, *Listerella* strains are all motile.

Comparison

Both *Listerella* and *Erysipelothrix* organisms are Gram-positive rods with no capsules or spores and have a similar arrangement in

PLATE III

FIG 1 —Smooth *Listerella* (58 XXIII), 24 hours at 37° C Gram ×915

FIG 2 —Rough *Listerella* (H Pine), 24 hours at 37° C Gram ×915

FIG 3 —Rough *Listerella* (H Pine), 24 hours at room temperature Gram
×915

FIG 4 —Intermediate *Erysipelothrix* (32), 24 hours at 37° C Gram ×915

FIG 5 —Smooth *Erysipelothrix* (33), 24 hours at 37° C Gram ×915

FIG 6 —Intermediate *Listerella* (53 XXIII), 9 hours at room temperature ×915

FIG 7 —Rough *Erysipelothrix* (9 35), 24 hours at room temperature Gram
×915

raised centre, flattened periphery, matt surface and slightly dentate edge and measure about 0.6-1.0 mm (fig 17) No change takes place on further incubation

Aerobic slope cultures at 37° C *Listerella*. After 24 hours, thin, smooth, confluent, raised, semi-transparent growth After 48 hours, growth is fairly profuse Rough forms show slightly spreading edge, but otherwise are similar

Erysipelothrix. After 24 hours, extremely poor growth, partly confluent, colourless, transparent, with slightly irregular surface and slightly dentate edge There is only slight improvement in growth on further incubation

Aerobic broth cultures at 37° C With both *Listerella* and *Erysipelothrix* growth in broth is similar except in degree The smooth strains show a uniform turbidity with slight deposit which disintegrates on shaking The rough strains show little or no turbidity, with thread-like masses of deposit which are difficult to disintegrate The amount of growth is much greater with *Listerella* than with *Erysipelothrix*

Gelatin slabs All *Listerella* strains showed a simple filiform growth Four of the *Erysipelothrix* strains showed a lamp-brush type of growth and the other two a filiform growth No liquefaction occurred

Loeffler's serum Growth is similar to that on agar

MacConkey's agar No growth

Peptone water *Listerella* strains grow fairly readily, but there was no growth of any of the *Erysipelothrix* strains unless serum was added

Resistance *Listerella* All strains survived moist heat at 55° C for 30 minutes but were killed after 60 minutes at this temperature

Erysipelothrix. Strains 33 and 6 35 were killed after 10 minutes and strains 32 and 9 35, Kolle and M S 3 after 20 minutes at 55° C (moist heat)

Metabolism *Listerella*. Aerobic only extremely slight growth under anaerobic conditions Growth favoured by glucose, slightly by blood or serum Optimum temperature for growth 30-37° C Growth takes place between 20 and 44° C

Erysipelothrix. Micro-aerophilic grows under aerobic and rather less well under anaerobic conditions Growth favoured by glucose, slightly by blood or serum Optimum temperature for growth 30-37° C Growth takes place from 20 to 42° C

Hæmolysin production is shown in table I

TABLE I
Hæmolysin production

Strains	Blood agar plates (48 hours)	Soluble hæmolysin
L 58 XXIII	+	+
L 53 XXIII	—	—
L H Pirie	+	+
L Schultz	+	+
L Gibson	+	+
E 32	Trace	—
E 33	"	Trace
E 6 35	"	—
E 9 35	"	—
E Kolle	"	—
E M S 3	"	—

For soluble hæmolysin a qualitative test only was carried out One c c of an 18-hour broth culture of each strain was mixed with 1 c c of washed horse red blood cells and the mixtures were incubated at 37° C for one hour

TABLE II *Biochemical characters.*

	LISTERELLA					ERYSIPELOTHRIX					
	58 XXIII	53 XXIII	H Pirie	Schultz	Gibson	32	33	6 35	9 35	Kolle	M S 3
Monosaccharides—											
Pentoses	—	—	—	—	—	—	—	—	—	—	—
Arabinose	A (tr)	A (tr)	A (tr)	A (tr)	A (tr)	—	—	—	—	—	—
Xylose	A	A	A	A (late)	A	—	—	—	—	—	—
Rhamnose	A	A	A	A	A	—	—	—	—	—	—
Hexoses											
Glucose	A	A	A	A	A	A	A	A	A	A	A
Lævulose	A	A	A	A	A	A	A	A	A	A	A
Galactose	—	—	—	—	—	A	A	A	—	—	—
Disaccharides—											
Sucrose	A (late)	A (late)	A (late)	A (late)	A (late)	—	—	—	—	—	—
Maltose	A	A	A	A	A	—	—	—	—	—	—
Lactose	A (late)	A (late)	A (late)	A (late)	A (late)	A	A	A	A	A	A
Trehalose	A	A	A	A	A	—	—	—	—	—	—
Trisaccharide—											
Raffinose	—	—	—	—	—	—	—	—	—	—	—
Polysaccharides—											
Starch	—	—	—	—	—	—	—	—	—	—	—
Inulin	—	—	—	—	—	—	—	—	—	—	—
Dextrin	—	A	A	A	A	—	—	—	—	—	—
Glycogen	—	—	—	—	—	—	—	—	—	—	—
Alcohols—											
Trihydric											
Glycerol	—	—	A (tr ?)	A (tr)	—	—	—	—	—	—	—
Hexahydric											
Mannitol	—	—	—	—	—	—	—	—	—	—	—
Dulcitol	—	—	—	—	—	—	—	—	—	—	—
Sorbitol	—	—	A (tr ?)	—	—	—	—	—	—	—	—
Glucoside—											
Salicin	A	A	A	A	A	—	—	—	—	—	—
Inosite	—	—	—	—	—	—	—	—	—	—	—
Litmus milk	A (tr)	A (tr)	A (tr)	A (tr)	A (tr)	A (tr)	A (tr)	A (tr)	A (tr)	—	A (tr)
Gelatin	—	—	—	—	—	—	—	—	—	—	—
Indole	—	—	—	—	—	—	—	—	—	—	—
Methyl red test	+	+	+	+	+	—	—	—	—	—	—
Methylene blue reduction	+ weak	+ weak	+ weak	+ weak	+ weak	—	—	—	—	—	—
Voges Proskauer reaction	+ weak	+	+	+ weak	+ weak	—	—	—	—	—	—
Ammonia	—	—	—	—	—	—	—	—	—	—	—
Hydrogen sulphide	—	—	—	—	—	—	—	—	tr	—	+
Catalase	—	—	—	—	—	—	—	—	—	—	—

TABLE III *Agglutination reactions*

Organism	Titre of antiserum prepared against									Normal serum
	<i>Listerella</i>					<i>Erysipelothrix</i>				
	58 XXIII	53 XXIII	H Pirie	Schultz	Gibson	32	33	6 35	Kolle	
<i>Listerella</i>										
58 XXIII	1 2500	1 1000	1 1000	1 5000	1 2500	1 20	1 10	0	1 5	1 50
53 XXIII	1 2500	1 1000	1 1000	1 5000	1 2500	1 25	0	1 25	1 25	1 50
H Pirie	1 2500	1 5000	1 1000	1 5000	1 1000	0	0	1 10	1 10	1 10
Schultz	1 2500	1 1000	1 1000	1 5000	1 2500	1 25	0	1 50	1 50	1 20
Gibson	1 2500	1 1000	1 1000	1 5000	1 2500	1 25	0	1 50	1 10	1 20
<i>Erysipelothrix</i>										
32	1 25	0	0	1 20	1 10	1 2500	1 1000	1 2500	1 2500	1 50
33	1 25	0	0	0	0	1 1000	1 5000	1 500	1 500	1 20
6 35	0	0	0	0	1 50	1 1000	1 1000	1 1000	1 2500	1 20
9 35	0	0	0	0	1 25	1 1000	1 1000	1 500	1 2500	1 20
Kolle	1 10	0	0	0	1 25	1 500	1 1000	1 250	1 1000	0
M S 3	0	0	0	0	1 25	1 1000	1 1000	1 500	1 1000	1 10

TABLE V
Leucocyte reactions

	Total W B C	Polymorphs		Lymphocytes		Monocytes	
		Per cent	Absolute	Per cent	Absolute	Per cent	Absolute
Strain L 58 XXIII 1000 million organisms per kg intravenously Rabbit 38/159							
Pre injection count *	11,000	42	4,620	56	6,160	3	330
After 84 hours	5,000	38	1,900	34	1,700	28	1,400
" 108 "	10,900	46	5,014	24	2,616	30	3,270
" 132 "	13,000	54	6,020	21	2,730	25	3,250
" 12 days	20,950	54	11,313	36	7,542	10	2,095
" 19 "	8,700	60	5,220	35	2,945	5	435
Strain E 32 300 million organisms per kg intravenously Rabbit 38/169							
Pre-injection count	16,400	30	4,920	65	10,680	5	820
After 110 hours	8,400	39	3,276	55	4,620	6	504
" 134 "	21,200	37	7,904	50	10,600	13	2,756
" 158 "	24,000	54	12,960	24	5,760	22	5,280
" 184 "	20,000	55	11,000	30	6,000	15	3,000
" 13 days	23,750	44	10,450	46	10,925	10	2,375
Rabbit paralysed—killed							
Strain E 33 1500 million organisms per kg intravenously Rabbit 38/93							
Pre-injection count	10,200	63	6,426	33	3,366	4	408
After 84 hours	16,500	43	7,095	50	8,250	7	1,155
" 108 "	10,900	38	4,142	38	4,142	24	2,616
" 132 "	13,900	50	6,950	39	5,321	11	1,529
" 9 days	29,000	52	15,080	34	9,860	14	4,060
" 48 "	11,500	45	5,175	51	5,865	3	345
Strain E Kolle 0.2 mg per kg intravenously Rabbit 37/31							
Pre-injection count	11,200	40	4,480	55	6,165	5	560
After 45 hours	11,050	29	3,200	60	6,630	11	1,220
" 65 "	19,500	46	8,970	42	8,190	12	2,340
" 112 "	13,200	34	4,488	55	7,260	11	1,452
" 204 "	14,600	39	5,694	44	6,424	17	2,482
" 16 days	15,000	54	8,100	43	6,450	3	450
Strain E M S 3 8,000 million organisms per kg intravenously Rabbit 38/94							
Pre-injection count	10,700	33	3,521	62	6,634	5	535
After 84 hours	13,600	37	5,032	52	7,072	11	1,496
" 132 "	12,700	39	4,953	45	5,715	16	2,032
" 156 "	13,000	37	4,810	52	6,760	11	1,430
" 22 days	12,200	46	5,612	45	5,490	9	1,098
" 35 "	12,400	25	3,100	70	8,680	5	620

* In all cases the pre injection count recorded is the one showing the highest monocyte count

(E 32 and 33) The doses were prepared as for mice and given intravenously. Two rabbits were given E 32 in doses of 1700 million and 500 million organisms per kg body weight respectively. Two were given E 33 in doses of 4000 million and 1800 million organisms per kg. All four died. Two rabbits were given smaller doses and survived. The disease did not differ appreciably from that occurring in mice. The organism was readily recovered from spleen and heart blood. No local lesion was produced. Conjunctivitis occurred in every fatal case. One or two focal lesions in the liver similar to those described in mice were seen. In two cases areas of mononuclear cell reaction occurred in the spleen resembling those seen in *Listerella* infection. Organisms were present in the liver, spleen and kidneys.

Guinea-pigs Doses were prepared as for mice and injected intraperitoneally.

Listerella Two guinea-pigs were injected with L Gibson in doses of 50 and 25 mg and 8 with L 58 XXIII in doses ranging from 40 to 2.5 mg. All died. The organisms were recovered from the spleen in every case and from the heart blood in some. *Post mortem* the changes resembled those seen in mice.

Erysipelothrix. Six guinea-pigs were injected with the most virulent strains of *Erysipelothrix* (E 32 and 33). All survived and showed no symptoms of disease, although large doses ranging from 30,000 million to 4000 million organisms were used.

Pigeons Doses were prepared as for mice and injected subcutaneously.

Listerella. Eight pigeons were injected with L 58 XXIII. Doses ranging from 100 to 5.4 mg were prepared from agar plates and from 150,000 million to 6000 million organisms (50-2 mg) from broth cultures. Three pigeons were given L Gibson in doses of 100 mg, 50 mg and 6000 million organisms (2 mg) respectively. All 11 pigeons survived and no symptoms of disease developed even after the enormous dose of 100 mg. One pigeon was injected with 200 mg of L 53 XXIII and died after 21 hours. Three pigeons were then injected with L 53 XXIII in doses of 200, 100 and 40 mg respectively. All three survived and showed no symptoms of disease. The one isolated death after only 21 hours was therefore assumed to be toxic and not due to *Listerella* infection.

Erysipelothrix. As the virulence of *Erysipelothrix* for pigeons is well known, I have used only E 32 and the mouse strain E M S 3. E 32 after one or two passages readily killed pigeons in doses of 100 million organisms. Four pigeons were injected with E M S 3 in doses of from 40,000 million to 9000 million organisms. All survived and showed no symptoms of disease. This strain was isolated in 1934 and is now relatively avirulent, the MLD for mice being about 1000 million organisms.

produce the two features which have aroused the most attention in *Listerella* infection in these animals, namely a circulating monocytosis in rabbits and areas of focal necrosis of the liver in rabbits and mice. In this connection it is of interest that Egehoj (1937) has recorded a circulating monocytosis in swine suffering from swine erysipelas.

Against these similarities must be set a fairly heavy list of differences. Within the limits of this study there appears to be no antigenic relationship between the two organisms. There are many significant differences as well as similarities in cultural characteristics and in metabolism. With regard to pathogenicity, *Listerella* readily kills guinea-pigs but apparently not pigeons, while *Erysipelothrix* readily kills pigeons but not guinea-pigs. The production of a circulating monocytosis in rabbits by both organisms is striking but it would be of interest to know if any other organisms have the same effect. Although focal necroses of the liver in rabbits and mice occur with *Erysipelothrix* they are an inconstant feature and, when present, are few in number, so that really the picture does not closely resemble that of *Listerella* infection. Finally the distribution and the naturally occurring diseases differ widely.

Conclusion

Five strains of *Listerella* and six of *Erysipelothrix* have been compared. In the present state of our knowledge it is impossible to decide finally upon their nomenclature and classification, but until further work has been done it seems wiser to retain the two generic names *Listerella* and *Erysipelothrix*.

This study was carried out at the suggestion of Professor R. A. Webb and under his direction. The work was finished at the Group Laboratory, Archway Hospital, and I am indebted to Dr J. M. Alston for affording me every facility. My thanks are also due to Professor G. S. Wilson for valuable criticism and suggestions.

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The following strains were used in this investigation and were obtained either direct from the persons who isolated them or through the courtesy of Drs Webb and Nyfeldt, Mr C A McGaughey and the National Collection of Type Cultures

- (a) Two strains of *L monocytogenes* (Murray *et al* , 1926 , Prie, 1927)
- (b) Four strains isolated from sheep , 101 G and 1378 G by Gill (1931, 1933), 12050 and 12053 by Jungherr (1937)
- (c) One strain from a goat isolated by Jungherr
- (d) One strain from a bovine isolated by Jones and Little (1934)
- (e) One strain from poultry isolated in America by Ten Broeck (Seastone, 1935)
- (f) Six strains from human beings, namely 4 strains isolated by Schultz *et al* (1933-34), Burn (1933-34), Gibson (1935) and Carey (1936) and 2 strains isolated by Nyfeldt in Denmark (personal communication)
- (g) Twelve strains isolated from poultry in England during 1937-38 by McGaughey (1), C V Watkins (4) and the writer (7) All these strains are referred to subsequently by the prefix LS

There is general agreement that organisms of the genus *Listerella* are motile and there is evidence that they possess a single polar flagellum when grown at 37° C Carey found that motility was most marked in seven-hour dextrose broth cultures grown at room temperature That a pathogenic organism may be more motile when cultured at a temperature lower than 37° C is a recognised fact *Pasteurella pseudotuberculosis*, which is actively motile at 22° C, is but sluggishly so at 37° C Arkwright (1927) and Schutze (1932b) used cultures grown at 18-22° C to demonstrate the presence of a flagellar antigen in this organism

McGaughey (personal communication) has shown that organisms of the genus *Listerella* are actively motile in dextrose broth when incubated at 25° C and has demonstrated peritrichous flagella (fig 1) in 12 strains of human and animal origin by the method described by Leifson (1930) The best results were obtained by doubling the amount of the saturated solution of basic fuchsin in ethyl alcohol and staining for several hours A counter-stain was not used McGaughey found further that rabbits injected with such cultures produced agglutinins and that a rapid, large-flake type of agglutination, similar to that observed with formalised suspensions of motile strains of *Salmonella*, occurred when the sera were tested against the appropriate bacterial emulsions

The writer has had ample opportunity of confirming McGaughey's results on a very much larger number of strains Maximum motility was observed in cultures 5-7 hours old, though 24-hour cultures

series starting at a dilution of 1 20 Readings were made after 30 minutes in the water-bath at 53° C No difference in the titre was detected if the time in the water-bath was increased or if the tubes were allowed to remain in the incubator overnight at 37° C

The results of these tests indicate that a serum prepared against any one of the strains of *Listerella* used in the experiment is capable of agglutinating all the other strains practically to titre A possible exception is the serum produced against the strain Jungherr 12053, which has significantly lower titres against the heterologous organisms than against its own antigen

Agglutinin absorption tests were carried out with 5 of the 16 mutually agglutinable strains, namely LS/2, Pirie, Jungherr 12053 Gibson and Murray

The bacterial emulsions used in the absorption tests were prepared as already described, in two-litre flasks After the addition of formalin (0.25 per cent), the growth was centrifuged (3000 *r p m* for 45 mins) and resuspended in saline to give an opacity six times as dense as tube 10 in Brown's scale Six ml of emulsion and 6 ml of serum diluted 1 10 were mixed in centrifuge tubes and heated at 53° C in the water-bath for 3 hours, the tubes being inverted once every hour After being kept in the cold store overnight the mixtures were centrifuged at 3000 *r p m* for 45 minutes The water-clear supernatant, now representing a dilution of 1 20, was decanted on to the deposit from 6 ml of bacterial emulsion After mixing, the heating, refrigeration and centrifugalisation were repeated This was found necessary because a single absorption did not produce complete removal of the homologous agglutinins The water-clear supernatants were set up against the agglutinable suspensions in the ordinary way

From a study of the results set out in table II it is apparent that the strains LS/2, Murray and Pirie are identical as far as their flagellar antigenic structure is concerned but differ from both Jungherr 12053 and Gibson, which in turn differ from each other

All the strains appear to have a common antigen LS/2 has an additional one which it shares with Jungherr 12053 but not with Gibson Gibson, on the other hand, has an antigen which is not shared by LS/2 nor by Jungherr 12053, while the latter possesses one not present in either LS/2 or Gibson Table III shows the structure as it appears from the analysis of the above results

Monospecific sera were now prepared in order to ascertain which of the antigens A, C, and D were present in each of the strains in our possession The combinations used to obtain these were as follows —

- For antigen A, LS/2 antiserum absorbed with Gibson strain,
- „ „ C, Jungherr 12053 antiserum absorbed with LS/2 strain,
- „ „ D, Gibson antiserum absorbed with LS/2 strain

The results of the agglutination tests obtained with these three

N. America and those from New Zealand with the exception of Schultz, which falls into group I. Gibson appears to represent a special group.

TABLE IV
Agglutination by whole sera and absorbed sera.

Antigen	Whole sera			Absorbed sera		
	LS/2	Jungherr 12053	Gibson	Antibody A	Antibody C	Antibody D
				LS/2 serum absorbed with Gibson strain	Jungherr 12053 serum absorbed with LS/2 strain	Gibson serum absorbed with LS/2 strain
LS/2	20,000	20,000	20,000*	640	0	0
LS/4	20,000*	10,000*	10,000	320	0	0
LS/5	10,000*	10,000*	5,000*	320	0	0
LS/6	10,000*	10,000	10,000	320	0	0
LS/7	10,000*	20,000	20,000	320*	0	0
LS/8	5,000*	5,000*	2,500*	320	0	0
LS/9	20,000	20,000	10,000	320	0	0
LS/10	40,000	40,000	40,000	320	0	0
LS/11	5,000*	10,000	5,000	320	0	0
LS/12	20,000	20,000	10,000	320	0	0
LS/14	10,000	10,000	10,000	160	0	0
Murray	20,000*	20,000	20,000	320	0	0
Pirie	40,000	20,000*	20,000	640	0	0
Nyfeldt/1 (CPMH)	20,000	20,000	20,000	320	0	0
Nyfeldt/2 (Ruth)	20,000	10,000	5,000	640	0	0
Schultz	2,500	5,000	2,500*	640	0	0
Jungherr 12050	10,000	20,000	20,000	160	160	0
Jungherr 12053	10,000	20,000	10,000	640	640	0
Jungherr (Goat)	20,000	20,000	20,000	160	160	0
Jones and Little	20,000	10,000	5,000	320	320	0
Ten Broeck	10,000	5,000	5,000	320*	640	0
Burn	20,000	20,000	20,000	320	320	0
LS/13	20,000	10,000	10,000	160	320	0
Gill 101	20,000	20,000	10,000	320	320	0
Gill 1378	20,000	20,000	20,000	320	320	0
Carey (O'Brien)	20,000	20,000	20,000	160	160	0
Gibson	20,000*	10,000	10,000	0	0	640

* = Partial agglutination in the next tube

In order to test further whether any other antibodies could be detected in the sera which had been prepared, a series of absorption tests were carried out at random. The resultant absorbed sera were used against LS/2, Jungherr 12053 and Gibson antigens, together with those concerned in each absorption test (table V). No additional antibodies were detected.

These results differ somewhat from those recorded by other workers. The probable explanation is that in the above investigations cultures grown at 25° C were used for the preparation of the antigens and therefore contained the maximum amount of flagellar antigen.

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controls or in 12 animals with only one sinus removed. Nevertheless the lesions they obtained appear from their description to be identical with those found by us in otherwise apparently normal rabbits.

Boyd and McCullagh (1937-38) have recently described aortic changes following bilateral denervation of the carotid sinuses. These lesions appear to be essentially the same as those described by Kremer *et al* and are considered by the authors to be dependent on the arterial hypertension consequent on the sinus denervation. They found arteriosclerotic lesions in 8 out of 12 denervated rabbits and state that pathological lesions were confined to these animals, without giving the number of their controls.

In view of the evident disagreement amongst workers as to the incidence of arterial changes occurring spontaneously in rabbits we have collected over the past 5 years hearts and aortas of rabbits killed in the course of a variety of experiments in this laboratory and have found a high incidence of aortic disease.

*Spontaneous aortic lesions observed in laboratory rabbits
during 1933-38*

Animals examined All the animals examined were, with three exceptions, bred and reared in the breeding rooms of the Sir William Dunn School of Pathology and, before being used in experiments, were apparently healthy. They were fed on the standard diet of bran or oats, hay, and mangolds or cabbage. All green foodstuffs were grown in the garden of the School of Pathology and picked fresh just before being eaten. The rabbits were used for a variety of experiments such as the injection of bacterial suspensions, externalisation of the carotid artery, etc., and were handled by many different workers. Most of the animals were killed at the completion of the experiments, only a few dying naturally. The age at death varied from 6 months to 3 years. Rabbits of both sexes were examined. The three exceptions above referred to were animals bought in the open market. One of these exhibited pronounced arteriosclerosis.

Incidence of aortic lesions The heart and aorta were removed and examined *post mortem*. The aorta was laid open from the aortic valves to the bifurcation of the common iliacs, these arteries being also examined. No macroscopic heart lesions were observed. Macroscopic changes were found in the aorta in 45 out of 144 specimens examined. The appearances of the lesions will be described later. It is necessary first to tabulate the histories of the animals in which these changes were found (table II).

In table III are given the histories of the rabbits in which there were no aortic changes.

TABLE III.

Summary of histories of animals without lesions of the aorta

A. General experiments					
Experiment			No of animals		
Normal animals			12		
Physiological experiments			13		
Injection of bacteria			12		
Injection of tyramine			14		
Other experiments			16		
Experiments involving the recording of the blood pressure (see B)			32		

B Experiments involving the recording of the blood pressure					
Animal no		Experiment	Blood pressure in mm Hg		
Serial	Laboratory		Normal	Rise of pressure	Duration of rise
22	1471	Bilateral excision of carotid sinuses	100	70	6 mths
23	2173	" " " "	85	40	Dropped to normal in 8 mths
24	2169	Nephrectomy	80	None	
25	2182	"	80	"	
26	2293	Injection of saline into renal artery	85	"	
27	2336	"	80	"	
28	1971	Injection of "Kieselguhr" into renal artery	80	40	6 mths
29	1972	" " " "	70	60	6 "
30	2058	" " " "	80	40	40 days
31	2078	" " " "	75	75	6 "
32	2089	" " " "	75	25	62 "
33	2168	" " " "	70	30	5 mths
34	1994	Injection of Kieselguhr into renal artery and excision of carotid sinuses	100	30	6 "
35	2185	Injection of Kieselguhr followed by stripping of renal pedicle	80	30	150 days
36	2186	" " " "	80	25	60 "
37	2188	" " " "	95	40	180 "
38	2189	" " " "	90	45	25 "
39	2200	" " " "	80	40	25 "
40	2266	" " " "	75	30	25 "
41	2187	Renal pedicle stripped followed by injection of Kieselguhr into renal artery	80	None	
42	2285	" " " "	85	"	
43	2288	" " " "	80	"	
44	2287	(a) Renal pedicle stripped followed by injection of Kieselguhr into renal artery	80	(a) None	
		(b) Renal artery constricted	80	(b) 30	2 mths
45	2178	Constriction of renal artery	75	40	18 days
46	2291	" " " "	85	30	80 "
47	2308	" " " "	75	40	10 "
48	2347	" " " "	75	35	55 "
49	2355	" " " "	75	30	50 "
50	2374	" " " "	95	None	
51	2380	" " " "	75	25	10 days
52	2108	Injection of nephrotoxic serum	80	30	30 "
53	2166	" " " "	75	30	75 "

Microscopic A large plaque is covered by intact intima. The media is practically absent as such and is replaced by tissue which we consider to be cartilage (see histochemical notes). There is a central area of necrosis in which there is no cellular structural detail. The elastic tissue and muscle have almost completely disappeared. The von Kossa stain for calcium is negative.

Rabbit 2054 (table II A common iliac artery tied, animal killed 6 hrs later) **Macroscopic** Small plaques about 3.0×2.0 mm scattered in first 20 mm of aorta. **Microscopic** The intima is intact and slightly hypertrophied over the lesion, which involves the inner third of the media immediately adjacent to the intima. Here the media is replaced by wavy bands of tissue which stain black with von Kossa's calcium stain. The wavy bands of tissue are irregular and broken and when stained with Weigert's elastic stain prove to be broken and distorted elastic fibres. The rest of the medial elastic tissue is also irregular and fragmented, and scattered throughout the media are small regions of hyalinisation of the muscular tissue. There appears to be no cellular infiltration.

Rabbit 2046 (table II A used for obtaining samples of normal blood) **Microscopic** There is a small area of cellular infiltration in the media lying immediately beneath a depression in the intima. The intima is intact. The nuclei of the cells in the infiltrated area resemble those of muscle cells, together with some resembling those of lymphocytes. With van Gieson's stain strands of hyaline material can be observed in the affected area. The elastic fibres are fragmented and in the central region of the lesion almost entirely absent. von Kossa's stain shows no evidence of calcium.

Histochemical notes on selected lesions

Two of the aortas (nos 1455 and 2203, nos 3 and 7 in table II B) contained the structures shown in figs 3 and 4. These plate-like formations in the media consist of a hard dense tissue, rich in intercellular substance and giving a negative reaction to the calcium test described below. The morphological similarity to cartilage is remarkable. In both there is a dense ground substance, often with dense capsular areas (fig 3) around the cells. The latter are large and vesicular and their nuclei contain one or more prominent nucleoli. Clear and refringent spaces are seen between the columns of cells (fig 4), these represent the attenuated elastic fibres of the media as evidenced by their staining with Weigert's elastic stain and orcein.

Although there is no specific histochemical test for cartilage, the metachromatic reactions of this substance are fairly characteristic. Thus it is stained purple with methylene blue or thionin, yellow with safranin, etc. Our material gave metachromatic reactions with these dyes as typical as those given by control specimens of young costal cartilage. Another feature in common with ordinary cartilage was the disappearance, partial or total, of the metachromasia on contact with alcohol and its return, provided differentiation had not been carried too far, on bringing the sections into water. Our sections were therefore examined either in water

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Stage 2 After three months the abdomen is again opened by a right paramedian crano-caudal incision well away from the former midline incision. The intra abdominal portion of the spleen is carefully removed after ligaturing all vessels, a strong thread ligature being tied around the spleen as close as possible to where it passes through the anterior abdominal wall (figs 2 and 3). The rat is thus left with an extra abdominal spleen about two-thirds its former size. Recovery is uneventful and rapid. The animals remain perfectly healthy, even for as long as 300 days afterwards. *Bartonella* infection does not appear, although our rats invariably develop this after complete splenectomy. We have had no failures in about seventy experiments. Adhesions sometimes form between the abdominal omentum and the divided end of the spleen, but normally the marsupialised spleen is supplied with blood from the subcutaneous and muscle vessels. India ink introduced into the circulation rapidly penetrates to the splenic pulp. Even when adhesions develop it is unlikely that the portal communications so established influence the spleen to any extent. We have had a number of animals without any abdominal adhesions and these form the basis of our conclusions. Nevertheless, animals with adhesions have not differed in the behaviour of their spleens from those without adhesions.

We have also studied splenic grafts implanted in the rat's peritoneal cavity or in the subcutaneous tissues. By 16 days a miniature spleen is formed and in young rats of 50-70 g it continues to grow until it may reach a diameter of 0.5 cm. Grafts appear to be more successful if splenectomy is performed. Some of these abdominal grafts become attached to the parietal peritoneum and like those in the subcutaneous tissues are independent of the portal circulation. It is possible therefore to have animals with splenic tissue still under the influence of the portal circulation as well as free from that influence (fig. 4).

We then produced progressive liver damage in (1) rats with marsupialised spleens or with splenic grafts, (2) rats with one-third or half their spleens removed, (3) normal rats. The animals were exposed daily for 6 hours in gas chambers to air containing 400-1000 *ppm* carbon tetrachloride, a concentration which produces fatal toxic cirrhosis with ascites in about two months. Some animals were killed 4-6 weeks after the commencement, others were allowed to go on until moribund. Pieces of liver and spleen were fixed in 70 per cent alcohol and paraffin sections stained with Ehrlich's acid haematoxylin and eosin, Weigert's iron haematoxylin and van Gieson, Laidlaw's modified Bielschowsky-Foot method for reticulum, Weigert's elastica method and the ferrocyanide-HCl-neutral red method for free iron.

Results

The normal rat's spleen The fully grown Wistar albino rat's spleen is enclosed in a capsule 15-25 μ thick, composed of 3-5 layers of collagen and elastic fibres with delicate reticulum strands between, becoming wider where trabeculae enter the pulp. Flattened elongated cells, often in groups but not uniformly arranged, lie amongst the fibres, together with cells closely resembling smooth muscle fibres. The capsule merges with the very thin peritoneal covering which has an outer lining of flattened mesothelial cells, easily rubbed off. Often elastic fibres are condensed beneath the mesothelium. There are very few capsular vessels except at the hilum, where the main vessels are congregated.

consist of a fine reticulum network with fenestrations between. There are no elastic or collagen fibres. A slight radiation of elastic fibrils from the trabeculae is often seen but this is soon lost. The pulp cells include (1) lining cells or reticular cells which resemble large lymphocytes, some of which contain brown amorphous or granular pigment giving the prussian blue reaction, (2) free sinus cells, including a few neutrophil and eosinophil leucocytes sometimes in clumps of three or four, a few lymphocytes, medium-sized monocytes, large macrophages 15-20 μ in diameter and numerous red corpuscles. Megakaryocytes are often seen in the young rat's spleen but are relatively few in adult animals.

The chief constituents of the *vascular system* are (1) penetrating arteries with much thick elastic tissue, two or three layers of muscle and a wide adventitia, (2) trabecular arteries, thin-walled in most instances, the larger showing several layers of smooth muscle and much elastic tissue in the adventitia, (3) follicular arterioles, described above, (4) fine terminal or pulp arterioles ending in the pulp, (5) venous or pulp sinuses described above, (6) veins of the pulp consisting of widely fenestrated endothelial tubes communicating with (7) trabecular veins whose walls cannot be separated from the trabecular tissue, (8) collecting veins of the hilum. We have not found any penicillar arteries with Schweigger-Seidel sheaths even after careful search of serial sections and are thus in agreement with Herrlinger (1938). Iron pigment is commonly met with in young rats but varies in amount in adults. It is generally found in the lining cells of the sinuses, the reticular cells of the pulp sinuses, occasionally in scattered macrophages and sometimes in clumps free in the pulp sinuses or around the Malpighian bodies. We have never seen it in close relationship to arteries or veins.

The marsupialised spleen Both in stage 1 and stage 2 spleens essential details are unaltered. The splenic capsule however is greatly thickened and carries a more elaborate vascular system. The pulp sinuses are well defined throughout. After some months the marsupialised spleen (stage 2) shows a progressive thickening of the reticulum fibres in the Malpighian bodies and red pulp.

The partly resected spleen Since there is reduction in bulk of the spleen during marsupialisation, we studied the spleen in control animals after removal of one-third to one-half of its substance. There was no departure from normal, except for the development of a fibrous scar with condensation of reticulum at the site of resection.

The spleen in control animals exposed to carbon tetrachloride We have confirmed most of Menon's (1938b) findings in normal rats given repeated subcutaneous injections of carbon tetrachloride. Small areas of necrosis were seen in the pulp and Malpighian bodies

PLATE XI

FIG 3 —The marsupialised spleen, stage 2, after 6 months. The skin has been reflected showing the spleen attached to the anterior abdominal wall muscles.

FIG 4 —Two months-old splenic grafts, one attached to the abdominal wall, the other to the small intestine. Complete splenectomy had been performed at the time of grafting.

changes were similar in the early stages, both in grafts under the influence of the portal circulation and free from that influence

Discussion

In recent years the idea has been growing that splenic changes associated with cirrhosis of the liver are due to two processes (1) pulp hyperplasia and (2) portal congestion. Early studies stressed the importance of portal congestion. Oestreich (1895) appears to have been the first to emphasise pulp hyperplasia. He described nine cases which seemed to bear out this conclusion. Actually only one of these can be considered satisfactory, a case of latent cirrhosis of the liver discovered at autopsy on a male with a fractured skull. There was no evidence of portal congestion and no sepsis. The other eight cases were complicated by infection. He concludes (p. 324) "*Das Wesen des Milztumors bei Lebercirrhose ist ein selbständiges, von der Leber unabhängiges und beruht auf irritativen Prozessen. Während der ersten Stadien der Cirrhose tritt eine zellige Proliferation (Hyperplasia pulpa) ein, welche auch bis in spätere Stadien hinein persistieren kann oder in mehr indurative Prozesse (Bindegewebsvermehrung) oder in wirkliche Atrophie der Pulpa übergeht*". The evidence for this view has been fully discussed by Eppinger (1920, 1937), Roessle (1930), Jager (1931, 1937) and McMichael (1934). The latter, in an important study of hepato-splenic fibrosis, clearly distinguished vascular changes due to increased portal pressure and showed that these were not the only features in the spleen in this disease. He stressed the importance of pulp hyperplasia in the splenomegaly. As evidence of hyperplasia he noted cellular increase in sinus walls and proliferation of active histiocytes into the lumen of the sinuses, activity being indicated by enlargement and a rounded appearance of the cells and by erythrophagocytosis. Similar changes were found in spleniculi. With portal congestion, the outstanding features were periarterial haemorrhages, periarterial fibrosis, siderotic nodules, perimallpighian haemorrhages or fibrosis with dilated venous sinuses and eventually increase of collagen in the sinus walls. There was no evidence of cellular proliferation in the pulp. He put forward the interesting view that this proliferation is related to hepatitis. Menon (1938 *a* and *b*) has recently studied splenic changes in experimental portal congestion and in acute and chronic liver damage. He, too, found no pulp hyperplasia with portal obstruction. On the other hand during the development of experimental liver cirrhosis proliferative and fibrotic reactions were seen in the spleen and splenomegaly developed before signs of portal stasis appeared. With chronic carbon tetrachloride intoxication Menon noted marked pulp hyperplasia coincident with the early

same as those used by Cameron and Karunaratne. Many normal animals kept under similar conditions served as controls. All experimental animals were chosen at random from the main population of rats.

Results

Acute liver damage Pentobarbital sodium given subcutaneously in doses of 40 mg per kg body weight to 55 normal rats induced sleep lasting over a mean period of 102 ± 4 minutes, the range being 25-210 minutes. Twenty-four hours after the administration of carbon tetrachloride, when the liver showed fairly extensive necrosis and fatty degeneration, the mean duration of sleep with the same doses of pentobarbital sodium in 20 rats was 218 ± 17 minutes, with a range of 80-435 minutes. There is a significant statistical difference between these means. Three to four days after the administration of carbon tetrachloride, when repair was going on actively by liver cell proliferation, the mean duration of sleep in the same group of rats was 98 ± 6 minutes, with a range of 30-184 minutes. There is no significant statistical difference between this value of the mean and that for normal rats. Seven days after carbon tetrachloride administration, when all the necrotic tissue had been removed and repair was nearly complete, the mean duration of sleep was 109 minutes. No deaths occurred in this experiment.

In another group of 19 rats, the mean duration of sleep after the standard dose of pentobarbital sodium was 108 ± 12 minutes. Tetrachloroethylene (C_2Cl_4) in amounts similar to those used in the carbon tetrachloride experiments was administered subcutaneously. Twenty-four hours afterwards, the mean duration of sleep after the same dose of pentobarbital sodium was 100 ± 5 minutes, no significant difference. The livers of all these animals showed no histological change. This experiment may be said to control the general intoxication induced by the administration of CCl_4 or C_2Cl_4 . In the one instance, however, there was acute liver damage and the duration of pentobarbital sleep was considerably increased. In the other case, there was no liver damage and the mean duration of sleep was not significantly altered. There appears to be a close connection, therefore, between acute liver damage and the action of pentobarbital sodium.

Evipan sodium, another quickly acting barbiturate believed by Weese (1933) and Kennedy and Narayana (1934-35) to be rapidly destroyed, mainly by the liver, gave a similar result. Thus in 12 normal rats, after 100 mg per kg body weight evipan sodium, the mean duration of sleep was 64 ± 6 minutes. Twenty-four hours after CCl_4 the mean duration of sleep in the same group of animals was 203 ± 14 minutes.

On the other hand, the slowly acting barbiturates barbital

After two months, 18 rats survived Pentobarbital administration now resulted in the death of 5 animals, a mortality rate of 28 per cent. The mean duration of sleep for the whole group, including the animals dying, was 208 ± 11 minutes (table). Excluding the 5 animals dying, the mean was 212 minutes. At this stage the liver shows more marked interinsular fibrosis, much regeneration but little degenerative change or necrosis. The liver weight, is if anything increased. The pentobarbital reaction is however considerably prolonged, whilst in 28 per cent of animals it is equivalent to a minimal lethal dose (120 mg per kg body weight), *i.e.* increased at least three times.

After two-and-a-half months there was considerable prolongation of sleep, even with still smaller doses of pentobarbital. Thus 20 mg per kg. body weight (half the standard dose) gave a mean duration of sleep in 10 normal rats of 60 ± 9 minutes, whereas with the 13 animals repeatedly injected with carbon tetrachloride it was 128 ± 16 minutes, a significant difference.

Three months after the commencement of the experiment, the animals had lost weight, some showed ascites and their livers were hard, coarsely nodular and yellow. Microscopically, bands of collagen and reticulum surrounded most of the lobules, condensation fibrosis was marked, regeneration nodules were small, whilst many of the lobules showed extensive areas of necrosis. The picture suggested a breakdown in compensation of a rapidly progressive toxic cirrhosis. At this stage, pentobarbital administration four days after the last injection of CCl_4 resulted in the death of 7 of the remaining 12 members of the group, *i.e.* a mortality rate of 58 per cent. The mean duration of sleep for the whole group was 249 ± 22 minutes (table), for the 5 survivors alone 319 minutes.

With progressive liver damage, therefore, there develops increased susceptibility to pentobarbital sodium and a mild physiological dose may become lethal.

Discussion

It appears from our experiments that rats with fairly extensive liver damage have an increased susceptibility to pentobarbital sodium and evipan sodium but not to barbital sodium and phenobarbital. This effect rapidly disappears when liver regeneration sets in. Both pentobarbital and evipan are known to be destroyed in the tissues, being excreted, in very small amounts only, in the urine and faeces. Barbital, on the other hand, is eliminated almost wholly by the kidneys, phenobarbital partly by the kidneys, partly through destruction in the tissues (Cushny, 1936). An acute intoxication such as that produced by C_2Cl_4 , a poison having many features in common with CCl_4 , does not alter the susceptibility

of such cases it would seem advisable to avoid trouble by the restricted use of this barbiturate

Summary.

Rats with acute liver damage are more susceptible than normal animals to the quickly acting barbiturates, pentobarbital sodium and evipan sodium. This effect quickly passes off when liver regeneration sets in. With progressive liver damage, as in the pre-cirrhotic stage of carbon tetrachloride cirrhosis, the action of pentobarbital sodium is greatly enhanced long before serious structural damage has appeared in the liver. It is suggested that impairment in detoxifying function precedes the development of cirrhosis. Need for caution in the use of these barbiturates is emphasised.

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1931, 1933, Dora Colebrook, 1935) and to show that streptococcal complications among patients in ear, nose and throat and scarlet fever wards are usually due to cross infection from other cases (Okell and Elliott, 1936; Allison and Brown, 1937) Brown and Allison (1937) found hæmolytic streptococci widespread in the air of scarlet fever wards, the types corresponding in many instances with those infecting patients in the ward, and thus supported Cruickshank's (1935) suggestion from the bacteriological investigation of burns that streptococcal infection may be spread by the dust or atmosphere of the ward

The serological typing of Streptococcus pyogenes

Preparation of agglutinating sera In the preparation of immune sera in rabbits I have followed Griffith's technique. Vaccines were made from all type strains by heating 400 c.c. of a 24-hour Hartley broth culture of the organism for 1 hour at 60° C., centrifuging it and resuspending the deposit in 20 c.c. of 0.85 per cent saline. The rabbits were given three intravenous injections weekly on successive days, beginning with the equivalent of 10 c.c. of culture. After the injections had been continued in increasing doses for 6 weeks, the final dose being equivalent to 20 c.c. of culture, the titre of the serum was tested. It was found that 16 out of 27 sera showed at this time a titre of 1:640, 6 a titre of 1:320 and 5 one between 1:80 and 1:160. Where the titre was low I have tried, unsuccessfully, to raise it by further injections, this procedure probably increases the production of heterologous agglutinins. It is important to watch the general health of the rabbits by regular weekly weighing throughout the period of inoculation. If there is any drop in weight injections should be stopped for a week. Only 2 of the inoculated rabbits died. A precaution adopted to minimise cross reactions was the use of individual syringes for each type throughout the period of inoculation.

Absorption of heterologous agglutinins The crude sera, after being tested against the homologous type, were tested against heterologous type strains in order to find out the content of heterologous antibodies. For absorption of these Griffith formerly used suspensions composed of all the heterologous type strains. Now he has simplified this method by using suspensions of one non-specific type 3 strain. Such a strain may be obtained as follows. A type 3 strain is plated on nutrient agar to which has been added 10 per cent of type 3 antiserum. Two varieties of colonies may be seen, one opaque, the other translucent or finely stippled. The opaque colony or segment of a colony is type-specific, the translucent colony is non-specific. Suspensions of a broth culture of such a translucent colony give no agglutination with type-specific type 3 antiserum and proved to be especially suitable for the absorption of non-specific antibodies. For this absorption 0.5 c.c. of crude type serum is diluted 1:5 with saline and mixed with heavy doses of the non-specific type 3 strain (usually the deposit of 500 c.c. of culture to 0.5 c.c. of crude serum). After centrifugation the supernatant serum is tested once more against the heterologous strains. The results are shown in the text figure.

It will be seen that certain sera were more prone than others to contain heterologous agglutinins and that all crude sera could be freed from non-type-specific antibodies by this procedure with the exception of the antisera to types 13, 17, 18 and 19. Absorption of these sera with suspensions of the type strains which had given cross agglutinations with the crude serum proved to be effective, except for the antiserum to type 17, which could not be freed of agglutinins to type 15.

Pauli and Coburn (1937) suggested that the cross agglutinations are due

The results, given in two series at the foot of table III, confirm the findings of Griffith and his co-workers that types 1, 2, 3 and 4 are predominant in scarlet fever cases. Certain changes from

TABLE III

*Percentage distribution of serological types of Str pyogenes among acute cases of scarlet fever in London, 1926-1937 **

Year	Number of observations	Type 1	Type 2	Type 3	Type 4	Types 1-4	Other identified types	Unidentified strains	Negative swabs
1926	100	2	34	17	23	76	0	17	7
1927	100	8	14	26	12	60	0	31	9
1928	200	9	25	17	5	56	0	36	8
1929	100	18	20	22	3	63	0	36	1
1930	100	17	19	19	7	62	0	35	3
1931	100	20	16	17	10	63	0	34	3
1932	100	25	10	7	1	43	36	21	0
1933	100	21	27	17	12	77	15	5	3
1934	183	7	25	16	16	64	22	7	7
1935	100	8	24	4	19	55	37	7	1
1936	141	13	15	9	13	50	38	6	6
1936-37 (Nov - May)	221	28.5	12.2	5.9	8.1	54.7	23.1	22.2	0.0
1937 (July-Nov)	200	19.5	10.0	5.5	8.0	43.0	20.5	33.0	0.0

* The data for the years 1926-1936 are taken from the Annual Report of the Chief Medical Officer to the Ministry of Health (1936). The serological typing was done by F. Griffith and later by V. D. Allison of the Ministry of Health's laboratories.

year to year will be noted in the incidence of these types. In our own series the lower percentage of types 1-4 in the second group may be associated with their occurrence in the inter-epidemic season, while the higher proportion of undetermined types as compared with Allison's findings may be related to the greater number of clinically atypical cases examined.

The relation of these four types to the clinical manifestations of scarlet fever has been studied by Gunn and Griffith. In comparing the incidence of faucial angina, vomiting and early adenitis, they came to the conclusion that the more severe forms of scarlet fever are associated with types 1 and 2, while types 3 and 4 produce milder forms of the disease. From an analysis of the clinical records of a small group of cases associated with types 1, 2, 3, 4 and 8, I found, in agreement with these authors, that early adenitis occurred most frequently in type 1 cases (table IV). As late complications may be due to cross infection, these are not included in the table. During the past winter an attempt has been made in collaboration with Dr M. J. Cooke to allocate patients with scarlet fever to particular wards according to the type of the infecting strain, with a view to eliminating cross infection and studying in more detail the natural history of the infection due to

more than one serological type is involved in an outbreak of streptococcal infection and it is therefore advisable to swab all the affected patients. This occurred in another outbreak of tonsillitis among the nursing staff and patients of a hospital to which a patient recovering from scarlet fever was admitted with a discharging ear. The first type isolated in this outbreak was type 2 and we were surprised on Dick-testing the nursing staff, that the Dick-positive members with tonsillitis had not developed scarlet fever. But further examination of their infecting types showed that type 25 was the causal organism, a type which rarely causes scarlet fever.

Puerperal sepsis The serological typing of *Str. pyogenes* from cases of puerperal sepsis may be of assistance in tracing the source and mode of spread of infection in sporadic cases and in epidemic outbreaks, in ascertaining whether certain serological types predominate in puerperal infections and in determining whether there is any correlation between serological type and severity of infection.

Source of infection Recent investigations have shown that the hæmolytic streptococci occasionally found in the genital tract of pregnant women (2.5 per cent) rarely belong to group A (Lancefield) and practically never initiate infection. The source of infection in puerperal sepsis has therefore to be sought outside the genital tract, either in the body of the mother or in those in close contact with her during or soon after confinement. Much publicity has been given to the attendant midwife or doctor as the carrier (nose, throat, or septic finger) of the infecting streptococcus, not enough, perhaps, to the mother as a possible source. Smith (1933) in his fuller series reported 9 out of 41 cases (21.9 per cent) where the infecting organism was recovered from an extragenital source in the patient herself, while in Dora Colebrook's series 12.5 per cent of the infections seemed to be autogenous. As access to outside contacts has not been possible in the present work an attempt is being made to find out how frequently the infecting streptococcus can be isolated from an extragenital source in the patient herself. For this purpose, nose and throat swabs are taken on admission of all patients to the puerperal unit at the North-Western Hospital and any septic foci present on the skin are bacteriologically examined. It is realised that the infecting organism may be carried after infection from genital tract to nose, throat or fingers, and in assessing which is likely to be the primary focus, we have taken careful account of the time relation of any upper respiratory tract infection to the puerperal fever, of the numbers of hæmolytic streptococci in nose or throat cultures and of the presence on admission of streptococcal antifibrinolytic substance in the patient's blood (indicative of a pre-existing streptococcal infection). On these standards, it was estimated that among 100 cases of puerperal

TABLE V
Distribution of types of Str. pyogenes in puerperal sepsis

Type *	1	2	3	4	5	6	8	9	10	11	12	13	14	15	17	19	22	25	26	27	28	Total typed	Total examined	Percentage typed
Colebrook	14	8	5	3	8	4	2	4	0	5	1	4	7	1	4	3	1	9	0	2	0	85	121	70.2
Shaw	1	4	1	4	3	5	3	3	1	6	1	1	1	1	0	0	3	4	0	2	3	14	79	55.7
Noisser	5	6	1	5	2	1	1	1	0	1	10	3	1	0	0	0	2	11	4	8	18	83	123	66.4
Total	20	18	7	12	13	10	6	8	1	12	12	8	9	2	4	3	6	24	4	12	21	212	325	65.2
Percentage of typed cases	9.4	8.5		5.7	6.1	4.7				5.7	5.7							11.3		5.7	9.9			

* There were no cases infected with types 18, 23 or 24. Cases from which types 7, 16, 20 or 21 have been isolated are omitted.

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(c) *Extract of killed and ground bacilli* The dried material prepared as in (b) was suspended in water in the proportion of 1 mg to 0.2 cc. This was centrifuged at 13,000 r.p.m. for about 45 minutes and the water-clear supernatant constituted the extract.

Titration of toxin

The toxicity of these preparations was compared by injecting guinea-pigs intravenously with freshly prepared material. A portion was dried and the dosage calculated as mg. of dry weight per 100 g. body weight. Three animals of 250-300 g. were used for each dose and in assessing potency the time till death as well as the number of deaths was considered. This method did not permit of exact determinations but indicated different degrees of toxicity.

The results with preparations from one set of cultures are shown in table I. The M.L.D. for living bacilli was of the order of 0.2 mg.,

TABLE I

Toxicity of preparations from Br. bronchiseptica

Intravenous injection of guinea pigs								
Preparation I Living bacilli			Preparation II Bacilli killed by freezing and thawing and ground			Preparation III Extract of II		
Dose (mg per 100 g.)	Deaths	Time till death	Dose	Deaths	Time till death	Dose	Deaths	Time till death
0.48	3/3	6-15 hrs	0.40	3/3	9 hrs	0.14	3/3	7-16 hrs
0.32	2/3	{ 1 in 6 hrs 1 in 24 hrs	0.30	3/3	9 "	0.07	3/3	7-16 "
0.16	3/3	{ 1 in 22 " 2 " 48 "	0.20	3/3	{ 2 in 9 hrs 1 " 10-19 hrs	0.035	3/3	7-16 "
0.08	1/3	4-5 days	0.10	3/3	10-19 hrs	0.02	3/3	{ 1 in 7-16 hrs 2 " 19 hrs
0.04	1/3	4-5 "	0.05	6/6	10-19 "	0.01	1/3	5 days
			0.025	1/3	50 hrs	0.004	0/3	
			0.012	0/3				

for killed and ground bacilli 0.05 mg. and for extract 0.02 mg. A second strain tested in the same way gave the same results. These doses were similar to those previously found for the corresponding preparations of *H. pertussis*. The lesions produced by each of these preparations were identical and were indistinguishable from those caused by *H. pertussis*.

Extracts made from four strains have been titrated frequently by intradermal injection of 0.2 cc. of a series of dilutions in water into the shaved back of the rabbit. The end-point was the highest dilution that caused a necrotic spot. There was some slight difference in the reactivity of individual rabbits but the usual

Formalin. Two separate portions of extract were formalised so that the final concentrations of formalin were 0·1 and 0·3 per cent. After incubating at 37° C for 20 hours the toxicity was determined by intradermal titration and it was found that the titre of the first was reduced from 1 1280 to 1 40 and that the second produced lesions undiluted. A control without formalin was incubated the same length of time and in this case the titre was reduced to 1 160. *Pertussis* toxin was also detoxified by 0·3 per cent. formalin in 20 hours at 37° C.

Antigenic property. The toxin of *Br. bronchiseptica* does not appear to be antigenic. Rabbits immunised over a period of 3 months with formalised toxin were as susceptible as normal rabbits to the intradermal injection of toxin. Their sera failed to neutralise 2 mrd of toxin *in vitro* (intradermal inoculation) although they contained agglutinating, precipitating and complement-fixing antibodies. These findings indicate that, as with *H. pertussis*, the extract contained bacterial antigen as well as toxin. The presence of bacterial antigen is also evident in later experiments in which the extract was used for precipitin and complement-fixing reactions.

Media in relation to production of toxin

Organisms grown on B G medium yielded extract of greater potency than those grown on 5 per cent horse blood agar or nutrient agar. Three freshly isolated strains were tested on each medium. Extracts were made as described and titrated intradermally in rabbits, the three preparations from each strain being tested on one rabbit (table III).

TABLE III

Media in relation to production of toxin

Medium	Titre (M R D) of toxin in rabbits		
	927	G P 1	G P 2
B G medium	1 2560	1 1280	1 2560
Blood agar	1 160	1 80	1 1280
Nutrient agar	1 320	1 80	1 640

The effect of B G medium on toxicity was further indicated by an experiment in which one of these strains was subcultured twelve times on agar. It was then grown on B G medium and on agar and an extract made from each growth. The intradermal titre of the first extract was 1 2560, the same as for the original culture,

whooping cough lesions We have confirmed their findings for *H pertussis* and further have obtained similar results with *Br bronchiseptica* A dose of 200×10^6 organisms killed 7 of 8 mice within 4 days, the survivor was killed after 9 days The organism was recovered in almost pure culture from the lungs of all The lungs of the mice which died had coalescing deep red patches in all lobes, some lobes being almost completely affected, the remainder of the lung was markedly congested and there was much frothy exudate Histologically the severity of the lesions varied somewhat, but there was marked general congestion, fluid exudate in many of the alveoli and oedema and cellular exudate in the alveolar walls In places, particularly around and extending from the bronchi, the alveolar spaces were almost obliterated by swollen alveolar walls compressed by exudate and cellular infiltration The epithelium of many of the bronchi was swollen and degenerated, there were scattered areas showing desquamation of cells and excess of mucus, with a varying amount of cellular infiltration The infiltration consisted of both polymorphonuclear cells and lymphocytes Clumps of small Gram-negative bacilli were found in the lumen among the bronchial epithelial cells and scattered throughout the lung in the alveolar walls The histological picture was similar to that seen in the lungs of mice infected with *H pertussis*

A dose of 10×10^6 *Br bronchiseptica* killed 2 of 6 mice in 4 days, the lungs were markedly involved and the organism was isolated from them The 4 survivors were killed after 7 days and although none of the lungs showed any notable gross lesions *Br bronchiseptica* was recovered from 2 of them This coincides with the findings for *pertussis* infection in mice, where the organism was recovered from lungs which showed no gross lesions in animals surviving up to 14 days

We were not successful in infecting guinea-pigs with *H pertussis* by intranasal instillation under anaesthesia Twelve animals were given a dose of 2500×10^6 organisms Two died, after 3 and 6 days, and the others were killed after 7 days None had any marked lung lesions and *H pertussis* was not recovered, but *Br bronchiseptica* was isolated from the lungs of 8 The incidence of *Br bronchiseptica* in these animals was about the same as in our normal stock, of 8 animals tested *Br bronchiseptica* was isolated from the lungs of 5

Guinea-pigs were inoculated with a similar dose of *Br bronchiseptica* The animals which died showed marked lung lesions like those produced in mice Histologically the lesions were usually more severe than in mice and the bacteria were present in larger numbers These lungs yielded *Br bronchiseptica* in culture as did the lungs without lesions from animals killed after 7 days.

6 or 7 weeks. One strain of each organism was used. Some sera were made against living and others against heat killed bacteria (table IV). No preservative was added to the sera, which were stored in the dry state. Living suspensions were made by scraping off the growth with a loop and emulsifying in saline. heat-killed suspensions by heating the living suspensions at 56° C. for 1 hour. The density of all suspensions was about 1000 million organisms per c.c.

Precipitation. Two sera made against living *H. pertussis* (P 59 and P 70) and two against heat-killed *Br. bronchiseptica* (B 59 and B 60) were used. The antigen was an extract made as described except that the ground dried bacilli were extracted with saline. All the sera reacted with each organism but the relative strengths of the sera were not determined.

Complement fixation. Five sera were used 2 made against living *H. pertussis* (P 69 and P 70) 1 against living *Br. bronchiseptica* (B 76) and 2 against heat-killed *Br. bronchiseptica* (B 59 and B 60). Two antigens of each organism were tested—heat-killed suspension containing 800×10^6 bacilli per c.c. and extract as made for the precipitin test in a dilution of 1/4. The technique of the reaction was described in our earlier paper. The guinea-pig complement was free from *bronchiseptica* agglutinins.

Each serum reacted equally with both forms of the homologous antigen, the titres of the different sera ranging from 1/80 to 1/640. Each serum also fixed complement with both forms of the heterologous antigen but to a lower titre. In heterologous reactions the suspension antigen was weaker than the extract antigen, the titres being 1/20 or less with suspension and 1/40 to 1/160 with extract.

We have previously shown that *pertussis* extract detoxified by heating at 56° C. for 30 mins. behaved in this reaction like unheated extract. This applies also to *bronchiseptica* extract.

Antigens prepared from *Br. bronchiseptica* grown on agar reacted in these tests to the same degree as antigens from organisms grown on B.G. medium. This fact, together with the difference in toxicity of organisms grown on these media, indicates that toxin and bacterial antigen are separate entities.

Agglutination. Sera made against living and heat-killed suspensions of each organism were tested with living and heat-killed suspensions prepared in the same way as those used for inoculation. Agglutination tests were incubated at 37° C. and read finally after 20 hours. The results are shown in table IV.

Pertussis sera agglutinated *Br. bronchiseptica* to $\frac{1}{4}$ or $\frac{1}{8}$ of the homologous titre. Three of the *bronchiseptica* sera agglutinated *H. pertussis* to $\frac{1}{4}$ of the homologous titre. Two *bronchiseptica* sera (B 59 and B 60) gave much greater cross reactions, agglutinating *H. pertussis* to $\frac{1}{2}$ and $\frac{1}{4}$ of the homologous titre.

The antigenic relationship was further investigated by heterologous absorption tests with heat-killed bacteria (table V), according to the same technique. Absorption was carried out separately with two strains of each organism and the absorbed sera were tested against living and heat-killed suspensions. Agglutinins for the heterologous organism were completely removed but the homologous titre was not affected or at most was slightly reduced. This applied also to sera B 59 and B 60 which showed much greater cross agglutination than the others. The results indicated that *H pertussis* and *Br bronchiseptica* each possesses a large factor of specific antigen and a small common factor, thus confirming the inference suggested by the results of cross agglutination.

Investigations to detect the possible role of a flagellar antigen of *Br bronchiseptica* in these results have indicated that the relationship between the organisms applies to the somatic antigen. The sera have at most contained merely a trace of flagellar antibody. *Bronchiseptica* sera absorbed with the homologous organism steamed for 1 or 2 hours or alcoholised removed all the agglutinins for steamed or alcoholised *bronchiseptica* suspensions, but the sera still agglutinated living suspension to $\frac{1}{4}$ or $\frac{1}{32}$ of the original titre. All the agglutinins for *H pertussis* were removed. The suggestion that the residual agglutinin corresponded to flagellar antigen was strengthened by the fact that absorption of a *pertussis* serum with steamed *H pertussis* removed all agglutinins.

Our results as regards the antigenic relationship of these organisms as shown by agglutination and agglutinin absorption are in general agreement with those of Eldering and Kendrick.

CULTIVATION AND MORPHOLOGY

On isolation, *Br bronchiseptica* grows readily on B G medium, giving in 24 hours colonies a little smaller than when grown on agar. The colonies are smooth, raised and glistening and resemble closely those of *H pertussis*, although they are much larger and the zone of hæmolysis is more intense and appears sooner.

We have confirmed the findings of Shibley and Hoelscher (1934) that *H pertussis* possesses a capsule and have shown that *Br bronchiseptica* possesses a similar one. The capsules of both organisms were difficult to stain and varied somewhat in size and shape. They were however quite definite and sometimes relatively large in comparison with the size of the organism. Frequently a small clump of organisms appeared to be imbedded in a mass of capsular material of irregular contour. There was some indication that the capsular substance was easily detached. Films made from heated suspensions showed fewer capsulated organisms and capsular material free from bacteria could be seen.

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have found certain discrepancies and ambiguities in the descriptions given by Clark and his co-workers

The construction of the chamber

The "round table" variety is the type of Clark chamber from which we started. The measurements worked out by Clark *et al* (1930) have been closely adhered to. The material employed is Perspex,* a very transparent plastic material, which can be turned on a lathe with very satisfying

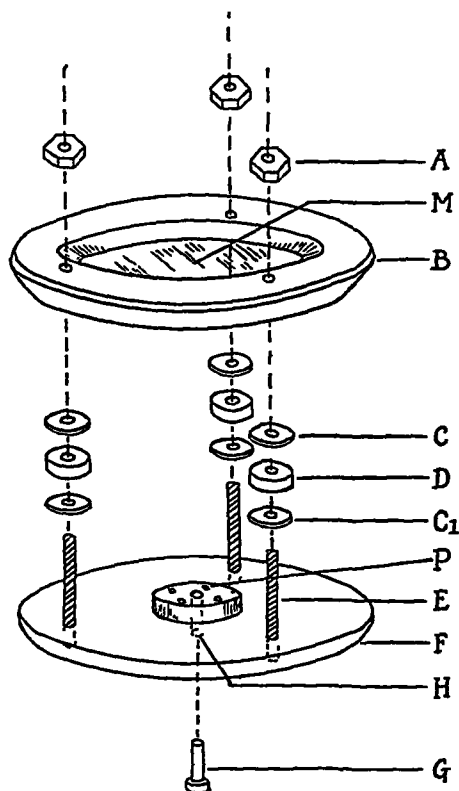


Fig 1 —Shows the separate parts of the chamber. Compare with fig 2

KEY TO FIGS 1 AND 2

- | | |
|------------------------------------|---|
| A, 10-B A brass nut | G, perspex plug (this plug has a shallow groove along one side) |
| B, perspex ring | H, hole to fit plug G, 1.115 mm |
| M, mica disc 0.5 mm | P, buffers 0.5 mm |
| C, C ₁ , rubber washers | R, radius for the three silver bolts and holes |
| D, celluloid washers | S, holes 1.8 mm diameter |
| E, 10 B A silver bolts | |
| F, perspex bottom plate | |

results and subsequently polished with metal polish. The base of the chamber (figs 1 and 2) with the central "table" is turned out as one piece. The top, consisting of a ring of perspex, has a sheet of thin mica cemented to it by means of Varian's cement (1931). Three silver bolts are fixed into the base to enable the top carrying the mica cover to be firmly fixed on

* This can be obtained from Imperial Chemical Industries, Thames House, London

The special feature of this design, besides its material, is the central plug to the table to enable particulate matter or grafts to be applied to newly formed tissue. This plug, which is very slightly less in diameter than the hole corresponding to it in the table, is made either to fit flush with the top of the table or to project above it for about 50-60 μ . The plug has a very shallow groove along one side to allow of air movement during manipulation. It is polished at both ends and is completely transparent. The handling of this plug will be described later.

Four perspex buffers are glued to the table top along the periphery. These hold the mica above the table and determine the thickness of the tissue which grows on to the central table. We have used buffers 50 μ thick. Perspex is soluble in chloroform, so that thin sheets of perspex can be made by pouring a little liquid perspex on to a clean piece of plate glass and spreading it out with the edge of another piece of glass. When the thin sheet has dried it can be removed with a little hot water. The edges of the sheet are cut away and discarded. The remainder is carefully measured with a micrometer, and regions of the appropriate thickness are cut out and used. The actual buffers are made by cutting pieces about 0.5 x 0.5 mm from a piece of the proper thickness with a sharp cataract knife. A dissecting microscope facilitates this operation. Shellac in alcohol is used to glue the buffers to the table, and a micro-pipette to apply the shellac to the region of the buffer. Since the shellac dries very rapidly, it is necessary to place the buffer on the glue as soon as a drop of shellac has touched the table. A dissecting microscope also makes this operation much easier. After the four buffers have been glued to the table the top is screwed on tightly so that the buffers are firmly fixed in place.

Insertion of the chamber

Four holes are punched in the rabbit's ear to take the central round table and the three silver bolts equipped with washers. An area of skin the size of the chamber is dissected away from the cartilage on both sides of the ear. The base is slipped into place and the top screwed to the base by means of nuts on the bolts. Although simple in principle, there are many pitfalls which may be encountered, and we believe a detailed account of the instruments used and the operative technique will be of value.

1 *Instruments used* (fig 3) (a) It is difficult to dissect the skin of the ear away from the cartilage in such a way that (i) the majority of the vessels on the cartilage are left intact and (ii) no epithelium is left behind. We tried many tools before finding one that was really satisfactory. The dissector used at present is a scalpel with a rounded tip ground to a dull edge.

(b) Clark and co-workers cut the holes for the central table with a knife and scissors. We have found it much simpler to punch them out with metal punches made for the purpose—a punch slightly larger (8.1 mm) than the central table is used for the central hole and another punch a little larger than the celluloid washers is used for the bolt holes.

(c) We have also found that we can get a much more accurate fit by using a thick perspex template for punching the holes. This template (fig 5) is drilled with four holes to correspond with the central table and the three bolts of the chamber. These holes are just large enough for the appropriate punches to slip through easily.

(d) The ear is held against a special wood block covered with lint moistened with saline while the holes are being punched.

(e) A heavy piece of brass is used to drive the punches through the ear.

(f) Special forceps are used for handling the central plug. Semicircular

slit and the cloth is clipped tightly to the base of the ear with skin clips. As a final precaution before starting the operation the ear is swabbed with cotton wool soaked in alcoholic acriflavine.

Before starting the actual dissection it is necessary to determine the future site of the chamber. It is advisable to place the chamber fairly near the tip of the ear to facilitate observation and to prevent the splints (to be discussed later) from cutting the skin, and the central table should be close to the central artery. Obviously there are two possible sites for the chamber since the edge of the table can be placed adjacent to either side of the artery. As a rule the chamber is inserted in position (a) (fig 4) with the

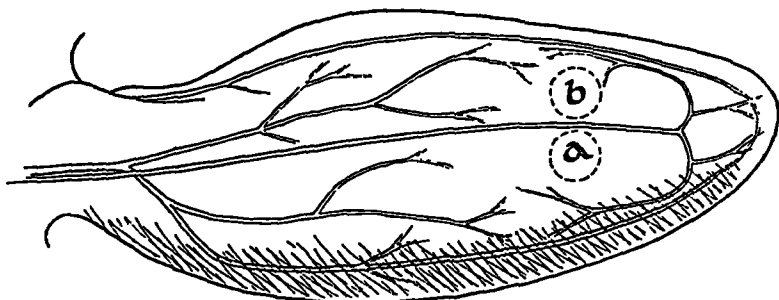


FIG 4—This illustrates the alternative sites for insertion of the central table $\times \frac{1}{2}$

central table adjacent to the edge of the artery nearest the rolled margin of the ear. The edge of the chamber sometimes presses against the curved portion of the ear, however, and may result in hypertrophy of the tissue along the edge. Therefore, if the ear is wide enough, it is desirable to place the chamber in position (b).

Having selected a site for the chamber the following steps are taken.

(a) **The holes are punched.** The template is placed over the site for the chamber on the outer surface of the ear, care being taken to avoid any large vessels in the regions to be punched. The wood block, covered with a piece of moistened gauze, is placed against the inner surface. The large punch is slipped into the central hole and, after making sure that the wood block is firmly gripped, the punch is given a sharp blow and the central hole is thus punched. Leaving the central punch in place, the smaller punch is slipped into each of the three peripheral holes in the template, corresponding to the position of the three bolts of the chamber, and each hole punched in the same manner. The large central punch is now removed. In this way an exact fit for the chamber is ensured. If care has been taken in avoiding large vessels there is little bleeding, and in any case bleeding usually stops after a minute or two.

(b) **Dissection.** There are now four holes cut through the cartilage and both layers of skin. It is necessary next to lift the two layers of skin from the region to be occupied by the chamber. Dissection is started from the edge of the central hole, on the inner surface of the ear, with a sharp-pointed probe. Having lifted the skin away from a narrow region of the cartilage the dissection is continued with the special dissector. The freed edge of skin is held with a pair of artery forceps and the rounded edge of the flat scalpel is firmly pushed between skin and cartilage. Dissection is thus carried out over a narrow radial strip of cartilage between the central hole and each of the bolt holes. The dissected area of skin is then cut in a radial direction with scissors or a knife. There are now three separate segments of skin to be lifted. The two nearest the base of the ear are dissected first.

splint is cut from a piece of celluloid 1.1 mm thick and is made in the shape seen in fig 5. We have found that it is wise to curve the edges of the inner splint to prevent cutting of the skin of the ear in the regions where there are natural folds. This is done by heating the celluloid in boiling water and bending it to the desired shape. Three holes are drilled in the celluloid (to fit 10-B A bolts), each hole being approximately 7 mm from the outer edge of the chamber when it is inserted. Shields to fit over these splints are cut from old X-ray film and are fastened with 10-B A nuts to the bolts holding the splints.

About a week before the insertion of the chamber, bolt holes are punched in the ear with a 2 mm punch, using the outer splint as a template. Before punching, the future site of the chamber is roughly determined with the aid of a celluloid model, and with the outer splint placed in position the splint holes are marked with a small punch which will fit through the small holes in the splint. The bolt holes themselves are then punched with the same punch, care being taken to avoid any large vessels. Usually there is very little bleeding. An ether anaesthetic is used for this operation and, as the procedure is short, the animal need only be kept under a few minutes.

After the chamber has been inserted, 10-B A bolts are passed through the holes previously punched in the ear and the two splints are bolted to those with 10-B A nuts. Perspex sleeves 5 mm long are fitted over the bolts before the splints are fastened in position and act as washers to prevent the splints from pressing directly against the ear. If these sleeves are not used the tissue around the bolt holes becomes greatly hypertrophied and often infected. Finally the protective shields are slipped on to the bolts and 10-B A nuts screwed on to keep them in position.

Observation of chambers

The rabbit is tied out unanaesthetised on the board designed by Clark, Sandison and Hou (1931). Sometimes for photographic purposes intravenous nembutal is given. A special metal plate covered with cork was made to fit a moving stage so that the ear could be clamped to it by spring clips applied to celluloid splints (fig 6). The exact position of the chamber is adjusted by pieces of plasticene so that the mica coverslip is parallel to the surface of the lens.

DESCRIPTION OF GROWTH AND NATURE OF TISSUE IN TRANSPARENT EAR CHAMBER

Since exhaustive studies have been made in Clark's laboratory on the growth of new tissue in similar chambers and on the nature of the organised tissue once it becomes stable, there is no need to give a detailed account of these points in the present paper, more particularly as our studies have only confirmed many of the observations of Clark *et al*.

General account of growth

Six to eight days after the operation capillary loops appear at the edge of the table, growing into the clot over the thin observation space. These vessels are always associated with and in many cases preceded by a row of large active macrophages which phagocytose the degenerating cells in the clot and clear the way for the advancing vessels. As a rule the first vessels to appear on the

the vessels. Still later a few smooth muscle cells can be seen along vessels which become arterioles. Certain capillaries near the edge of the table which are more favourably placed for supply and drainage of the transparent area become transformed into arteriolar and venous capillaries, and later a few of these become the arterioles and venules which handle the greater part of the blood supply on the table. If vessels have come in most of the way round the central table, vascularisation is completed by the anastomosis of vessels near the centre of the observation table.

Once the chamber has been completely vascularised certain changes occur before the observation area assumes what Clark terms "the adult pattern".

1 *Disappearance of free fluid* Generally there is free fluid in the growing chamber, particularly in the region of the growing edge, and it can be identified by the pulsation of extravascular blood cells. Once the chamber has been completely vascularised the free fluid disappears. Clark believes that the intercellular substance of an "adult" chamber is a gel which is only liquified under certain abnormal conditions, such as endothelial injury or infection, and our observations support this view.

2 *Growth of vessels* After some weeks the number of vessels on the central table is greatly decreased and a small number of vessels become larger and straighter and are transformed into arterioles or venules which handle the main supply of blood to the tissue of the observation area. Arterioles can be identified by their narrow lumina, thicker endothelium and muscular walls and rapid circulation, while the venules appear as wide vessels with thinner walls and slower circulation. Many of the original capillaries are resorbed, while some of the others show active circulation only occasionally.

3 *Reduction in number of extravascular cells* A fresh hæmorrhage immediately in front of the growing edge is usually seen in the new chamber. These extravascular red cells, as well as polymorphs and non-granular leucocytes which have migrated out of capillary loops, gradually disappear after the tissue in the chamber becomes more stable. Once the original clot has been cleared away the large macrophages which accompanied the capillary sprouts also disappear, so that the only cells normally found in the tissue of the "adult" chamber, apart from a few polymorphs, are histiocytes and fibroblasts. The histiocytes are particularly numerous along the larger vessels.

4 *Increase in connective tissue stroma* Accompanying these other changes there is a great increase in connective tissue fibres as the chamber grows older. Connective tissue fibres are laid down as soon as a region is vascularised, and as the new tissue becomes more stable the density of the stroma increases.

At first, attempts were made to scrape out abscesses, but without much success. Even if a large part of the abscess is removed a few cocci are left behind and the abscess starts to re-form. It was then decided that the chamber might be removed, the dissected area cleaned up and a new chamber inserted. It was found that this technique met with considerable success.

Technique A new chamber and instruments are sterilised as before. The rabbit is given nembutal intravenously and the chamber is removed outside the operating theatre. If there is an infection the pus is wiped away before sterilising the ear. Keratinised epithelium usually adheres to the chamber when it is removed, but if any is left behind this is also scraped away. The ear is then soaked in a watery solution of acriflavine for half an hour.

The remainder of the "repair" is done in the theatre with complete aseptic precautions. If epithelium is present it is dissected away with the special dissector and the top layer of new connective tissue is removed as well. Before inserting the new chamber the edge of the central hole is trimmed with scissors. Unless the vessels along the edge of the hole are injured in this way there is no growth of new vessels on to the central table. The insertion of the new chamber is quite simple since there are no skin flaps to pull over the edges of the chamber. It has been our experience that more harm than good is done by sealing the edges of "repaired" chambers with celloidin and cotton.

All repairs so far done have been successful. In one, the growth has been extremely slow, since a wide space was left between the cartilage and the central table. Practically no injury is done to the vessels in the cartilage during the second operation.

In general the "repaired" chambers are more satisfactory than the original in that they remain freer from infection at the edges and give an excellent growth on the table.

By utilising this "repair" technique it is possible to use one rabbit for several experiments—a point of importance, as it gets accustomed to being handled and observed.

Technique and effects of pulling out the central plug

Two types of central plug have been used, as already mentioned.

(1) a plug which is flush with the central table, and (2) a plug which projects 50-60 μ above the table top and comes in contact with the mica coverslip. In both cases the region of the central plug is quite as transparent as the rest of the central table.

1 *Growth in the two types of chamber* (a) *Central plug flush with the table top* When the growing vessels approach the centre of the table they bridge the small gap of a few μ between the table and the central plug and invade the region above the central plug in the usual way. In course of time vessels anastomose on the plug and it becomes vascularised in the same manner as the rest of the table. The presence of the central plug does not seem to modify either the rate of growth or the ultimate vascular pattern of the chamber.

to the tissue by removing the central plug and pushing in a new one. Since much of the success of the operation depends on the strength of the connective tissue, however, it is wise to wait until several weeks after the chamber has been completely vascularised before attempting to pull out the plug. Practically the only damage done seems to result from the initial movement of the plug away from the tissue over the central hole. As long as the plug is removed slowly the vessels in this region remain comparatively uninjured. Apparently injury may be done to the vessels bridging the gap between the table and the central plug. One or two may be nipped when the plug is started out of the central hole and slight hæmorrhage may result. Almost no injury is done to the vessels away from the periphery of the plug.

Naturally the picture after the plug has been replaced depends upon the amount of damage done during the operation. When this is slight, the area over the central plug appears much the same as it did before the plug was removed. One or two vessels near the edge of the plug may be in temporary stasis, but circulation starts again in a few hours if the vessels were not badly injured. The endothelium of most of the vessels on the central plug exhibits stickiness to leucocytes of the blood stream, and there is usually migration of a small number of leucocytes, mostly polymorphs, in the first 2 or 3 hours after the operation. The endothelium in some of the vessels may remain sticky for several days, but migration of leucocytes only occurs during the first few hours. In the case of small hæmorrhages near the edge of the plug the extravasated blood does not extend into tissues very far on either side of the gap between plug and table. These small hæmorrhages are gradually cleared away by macrophages, and although the process may take as long as 5 or 6 days the reaction is entirely a local one. The tissue on the central plug may remain rather more cellular than normal "adult" tissue, due to the presence of leucocytes, but these disappear during the course of 5 or 6 days.

Vaseline was accidentally pushed into one chamber along one side of the central plug and this was ingested very slowly by macrophages (figs 10 and 11). Again the reaction was entirely a local one, limited to the region of the vaseline, and the remainder of the tissue on the central plug was not obscured by its presence.

In one case the original central plug was much too tight and a good deal of damage was done to the vessels on one half of the table when it was pulled out. In this case the reaction was rather different. On examination after the plug had been pulled out it was seen that half the vessels on the central plug were in stasis, and that there had been extensive hæmorrhage both on to the central plug and into the surrounding tissue. Many vessels were in only temporary stasis and, after several hours, circulation

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ethylene was combined with testosterone propionate (11 out of 14 animals or 79 per cent) Allowing for differences in the degree of mucification it is permissible to conclude that the addition of testosterone was more effective than the addition of progesterone The mucification produced under these experimental conditions is illustrated in figs 1-4

Experiments on guinea-pigs. Five guinea-pigs received twice-daily injections of 20 mg of triphenyl ethylene in 0.1 c c of oil Nos 1b and 1c were injected over a period of 13 days and killed 3 days after the last injection, nos. 2a, 2b and 2c were injected over a period of 11 days and killed 2 days after the last injection In all these animals a high degree of mucification was found (fig 5) A similar degree of mucification was also found in two control animals, 3b and 3c, which received twice-daily injections of 10 microgrammes of oestradiol benzoate in 1 c c. of oil over a period of 11 days and were killed on the day after the last injection

In all these seven animals blood was present in the vagina during the last few days preceding the post-mortem examination The blood originated in the uterus It is hoped to discuss this phenomenon in a future communication

Summary

Previous investigations have shown that the synthetic oestrogen, triphenyl ethylene, not only produces vaginal keratinisation in the lower rodents but also a series of changes in the bitch, rabbit and monkey similar to those produced by the natural oestrogens The present experiments show that triphenyl ethylene will also produce vaginal mucification similar to that produced by small doses of oestrin and that as in the case of the natural oestrogen the mucification is increased when progesterone or testosterone is given at the same time

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PLATE XV

FIGS 1 4 —VAGINAL MUCIFICATION IN MICE

FIG 1 —Mouse MU 15b, g, injected with 0.5 mg triphenyl ethylene Mucification ++ ×300

FIG 2 —Mouse MU 8b, injected with 0.06 mg triphenyl ethylene Mucification + ×300

FIG 3 —Mouse MU 14h, injected with 0.5 mg triphenyl ethylene and 1 mg progesterone Mucification +++ ×300

FIG 4 —Mouse MU 13h, injected with 0.5 mg triphenyl ethylene and 0.25 mg testosterone propionate Mucification +++ ×300

FIG 5 —Vaginal mucification in guinea-pig GP 1b, injected with triphenyl ethylene ×75

the rejection of the terms *gravis*, *mitis* and *intermedius* because the clinical implications which they convey are unjustified

It is not our intention in this paper to attempt to review this new literature, but to point out that there is a conflict of opinion, not about the existence of the three types described, but about the validity of the suggestion that the rough starch-fermenting diphtheria bacillus is a much more serious menace to the person infected than the smooth non-starch-fermenting variety

There are obvious weaknesses in some of the criticisms advanced, e.g. the practice of using one only of the criteria recommended for the differentiation of the types, namely the appearance of colonies on solid media (Hilgers and Thoenes, 1936 *a* and *b*). It is often inferred that the observation of a number of mild cases of *gravis* infection effectively disposes of any justification for calling the bacillus by that name. It is noteworthy that von Bormann, in an extensive review of work bearing on the significance of the diphtheria types, first deprecates the practice of typing based on one characteristic only, and then quotes the work of Hilgers and Thoenes, which is so based, as providing definite ground for rejecting the classification into *gravis* and *mitis* types

It would be absurdly out of keeping with all we know of infective disease to claim for any one variety of micro-organism that all cases of infection by it were severe. No such claim has ever been made for the *gravis* type of diphtheria bacillus. It seemed to us, however, that we would be more likely to elucidate the matter by seeking further evidence for or against the hypothesis advanced than by a detailed criticism of the published work. We have been engaged, therefore, during the last two-and-a-half years in an investigation of the post-mortem appearances in fatal cases following infection with the various bacillary types

The chief obstacle to the progress of this investigation has been the infrequent occurrence of *mitis* deaths. The *gravis* epidemic reached its height in Leeds in the year 1934. Since then this type of infection has gradually receded and *mitis* infection has become predominant, without ever being responsible for so high a percentage of all cases or so large a total of cases as were associated with predominant *gravis* infection between 1931 and 1935. These fluctuations are indicated in fig. 1. The trend of diphtheria from 1935 to midsummer 1938—a rising incidence of *mitis* infection—has therefore been optimal for the observation of severe cases of *mitis* infection and deaths from such infections have in fact been a good deal more numerous than in the period 1931-34. This notwithstanding, our observations on the post-mortem appearances in these infections have accumulated very slowly. Throughout the last seven years infections with the *intermedius* type of the diphtheria bacillus have been rare in Leeds. This is not easily

death The cultures together with material for histological examination have been sent to Leeds from each of these cases

Including the latter, our series consists of 51 cases, of which 23 were *gravis* infections, 14 *intermedius*, 11 *mitis* and 3 mixed (2 with *gravis* and *mitis* strains and 1 with *intermedius ante mortem* followed by *mitis post mortem*) An important point in this

TABLE I *Diphtheria in R School*

Date	Case	Severity
1 6 37	G { E W I W M W	Slight
3 6 37		"
3 6 37		"
29 7 37	I { S H E W J H J E H L M P I G P M G D C E A N L M B N P H H D S	Moderate
12 10 37		"
4 11 37		Severe (died)
22 11 37		" "
23 11 37		Moderate
24 11 37		"
6 12 37		Severe (died)
13 12 37		Moderate
24 12 37		Severe (died)
25 12 37		Moderate
28 12 37		"
28 12 37		Severe (died)
3 1 38		" "

The first three cases, all from the same family, were *gravis* infections Eight weeks after the removal from school of the last infected member of this family an *intermedius* infection appeared From then onwards for six months only *intermedius* infections were recorded in this school The severity of these infections, however, was remarkable The post-mortem findings show that double infections with *C diphtheriae* and *H influenzae* were present in most if not all of these cases and may have been partly responsible for their unusual severity

investigation seemed to us to be that the type determination recorded during life for each case should be confirmed by the post-mortem isolation of the same type of diphtheria bacillus if the findings at necropsy were to be accepted as reliable examples of the morbid changes associated with that type

In this connection it is of interest to note Preuner's (1936-37) suggestion that some of the recorded cases of *mitis* death may in fact be due to cross infection in the wards of a fever hospital Further, it may be significant that Hilgers and Thoenes, who record a much higher death rate in *mitis* diphtheria than any other writers on the subject, also describe an unusually high incidence of type changes on repeated swabbing and of mixed infections. Before entering on our results, however, it is desirable to describe briefly the methods employed

SCOPE AND METHODS OF BACTERIOLOGICAL OBSERVATIONS

In most instances a culture taken before death was examined In all such cultures as well as in those taken after death the following points were noted in determining the type of *C diphtheriae* recovered

(b) *Agreement of findings before and after death in respect of bacterial type*

Our observations on this point were limited in scope owing to the absence of a bacteriological examination prior to death in seven cases and failure to isolate diphtheria bacilli from the body in six cases which died late. There remain, however, 38 cases (16 *gravis*, 13 *intermedius* and 9 *mitis*) in which the bacteriological type was found to be identical on examination in life and after death. In three cases a discrepancy between ante-mortem and post-mortem findings was recorded (table III). The first two cases

TABLE III

Three cases showing a discrepancy between the ante-mortem and post-mortem bacteriological findings

Place of origin	Bacteriological findings	
	during life	after death
Leeds	<i>Gravis</i>	<i>Gravis</i> copious, <i>mitis</i> numerous
Liverpool	<i>Intermedius</i>	<i>Mitis</i> death 7 weeks after first examination
Liverpool	<i>Mitis</i>	<i>Gravis</i> predominant, <i>mitis</i> numerous, both pathogenic to guinea-pigs

might readily be explained by cross infection in the wards. The findings in the third case are not easily interpreted. A mixed culture of *gravis* and *mitis* was obtained from the throat. One colony of *gravis* was recovered from the left lung and a small number of colonies of diphtheria bacilli appeared in cultures from the right lung. Amongst these the presence of (a) *mitis* resembling that found in the throat and (b) *gravis*, was determined.

There were 28 cases in which *C. diphtheriae* was recovered from both tonsils and lungs *post mortem* (15 *gravis*, 9 *intermedius*, 3 *mitis* and 1 *gravis*+*mitis*). In all these cases the type of bacillus recovered from the lungs was identical with that recovered from the local lesion.

OBSERVATIONS ON THE PRESENCE OF OTHER PATHOGENIC ORGANISMS AND THEIR POSSIBLE INFLUENCE ON THE FATAL ISSUE

Attention was concentrated particularly on the presence of bacteria other than *C. diphtheriae* in the spleen and lungs in order to determine (a) whether secondary infection played an important part in causing death, (b) whether secondary infection was more important in the deaths due to one or other of the diphtheria types

MORBID ANATOMY

It will be most convenient to deal in the first place with the 48 cases in which one and the same type of organism was present at all bacteriological examinations, both *ante* and *post mortem*

Local lesion

The primary local lesion was situated in the fauces in most cases, there were six exceptions, however, namely one *mitis* (nasopharynx), three *gravis* (nose 2 larynx 1) and two *intermedius* (nose 1 larynx 1) In the latter there was no involvement of the fauces and with the exception of one nasal *gravis* case they all showed persisting evidence of acute diphtheritic inflammation at necropsy

Of the 42 cases with primary faucial lesions, 31 died in the acute phase of disease and showed corresponding change in the tissues of the throat Included in this group are 7 *mitis*, 15 *gravis* and 9 *intermedius* cases Resolution of the local lesion was complete or almost so in 6 cases (3 *mitis* and 3 *gravis*) while in the remaining 5 cases (3 *intermedius* and 2 *gravis*) subacute or chronic inflammation with fibrotic changes was found

Perhaps the most important observations arising out of the present investigations refer to the differences in the acute local lesion in typical *mitis* and *gravis* diphtheria Before describing these in detail, it may be well to state that importance is attached to two points in particular, (1) the much deeper penetration of the tissues below the surface in *gravis* as opposed to *mitis* infections, and (2) the marked selective effect on the tonsils and cervical lymphatic glands in *gravis* and *intermedius* diphtheria Such findings make it possible in the majority of cases to distinguish between *mitis* infection and infection with the other two types before the organism itself is identified No distinction can be made on these grounds between *gravis* and *intermedius* diphtheria

Mitis diphtheria Here there is generally an abundant formation of firm cohesive membrane which may involve the faucial pillars, tonsils, uvula, soft palate, posterior pharyngeal wall, base of tongue and epiglottis In a considerable proportion of cases (table V) it spreads downwards into the larynx, trachea, bronchi and even for some distance into the intrapulmonary branches of the last named In the throat, the tonsils are never involved to a greater degree than the surrounding structures, indeed they may sometimes be relatively free The cervical glands show only minimal enlargement

Histologically the membrane is found to consist of abundant fibrin, in the meshes of which are entangled a considerable number of leucocytes and a few red blood cells The epithelial surfaces

clinical experience, the *gravis* infections in this series frequently showed very large amounts of membrane. At necropsy, however, it has not been a conspicuous feature. This paradox will be discussed later, in the meantime it should be emphasised that references to membrane formation in the ensuing descriptive section apply only to our post-mortem findings. In *gravis* diphtheria we have rarely found any considerable formation of membrane except in relation to the enormously enlarged tonsils, where the superficial exudate is continuous with similar material extending deeply into the substance of the gland. When membrane is found in other situations, it is only exceptionally of the tough cohesive texture of *mitis* membrane and is generally friable and granular or mucoid, breaking up when attempts are made to detach fragments. Only infrequently does it reach the larynx and upper trachea, and in the present series never the lower trachea and bronchi.

The tonsils are enlarged, sometimes to a very great size (fig 4). Their surfaces are rough and ragged, and covered with necrotic and often malodorous material. The colour is variable: when only a short interval has elapsed between death and necropsy they are a rich purplish red, otherwise they are most often almost black. In size, colour and texture the intact tonsils have sometimes recalled the appearance of a pickled walnut. On section the same colour may be seen throughout, but more often the cut surfaces are mottled or laminated and streaked with yellow. Haemorrhage can be seen in the tonsillar bed.

Histologically, almost all the lymphoid substance of the tonsil is replaced by exudate which varies in composition from place to place (fig 2). Sometimes there is a considerable amount of fibrin, though never so much as in the typical *mitis* membrane and it is always more heavily infiltrated with leucocytes, in parts the exudate is frankly purulent, the leucocytes being degenerate and fibrin absent, while in other parts there are quite extensive hæmorrhagic areas containing few cells except red blood corpuscles. Only the merest remnants of lymphoid tissue remain. In the tonsillar bed there are irregular hæmorrhages, leucocytic infiltration, inflammatory cedema, intravascular thrombosis and patchy necrosis of voluntary muscle fibres. In the more chronic cases collagenous scars can be found both within the tonsillar substance and in the subtonsillar tissues. Recovery and regeneration of the tonsil is evidently a more protracted process in *gravis* than in *mitis* diphtheria.

While the tonsillar phenomena constitute the most striking part of the *gravis* lesion, there is generally considerable swelling of the surrounding tissues, including the posterior pharyngeal wall. In a high proportion of cases there is cedema of the ary-epiglottic folds, which is at any rate in part inflammatory in origin. The

necrosis" Its incidence in the various types of case is shown in table VI

TABLE VI

Incidence of hæmorrhagic necrosis of the tonsils

	<i>Gravis</i>	<i>Intermedius</i>	<i>Mitis</i>
Well marked acute lesion	14	7	0
Chronic or subacute lesion with necrosis and fibrosis	4	5	0
No lesion	3	1	10
Total	21	13	10

Four cases are excluded from this table one recent tonsillectomy and three infants with very rudimentary tonsils

Larynx, trachea and bronchi

Many of the important findings in these situations have already been dealt with The primary lesions were laryngeal in two cases, one *gravis* and one *intermedius*, but extension of the superficial membrane to the air passages from a faucial primary lesion was much commoner in *mitis* infections than in the other two (table V) The firmer character of *mitis* membrane was particularly well shown in these sites Tracheotomy had been necessary in five *mitis*, three *gravis* and two *intermedius* cases respiratory obstruction was the most frequent cause of death in *mitis* cases but this was relatively rare in *gravis* and *intermedius* cases

In a number of *gravis* cases the trachea and bronchi contained large quantities of dirty brown, rather thin mucus of unpleasant odour A degree of mucous bronchitis was almost constant throughout the series, mucopurulent and purulent bronchitis were more frequent in *gravis* and *intermedius* cases

Lungs

The most constant finding was acute general emphysema, this was present even in cases which did not show clinical signs of respiratory obstruction The condition was generally accompanied by small areas of collapse, especially at the posterior surfaces of the lower lobes

Bronchopneumonia was found in a high proportion of cases and may be roughly subdivided into three categories

- (1) Aspiration pneumonia (in late paralytic deaths), one *gravis*, one *mitis* 2 cases
- (2) Severe, sometimes confluent bronchopneumonia, probably of significance in relation to death, three *gravis*, three *intermedius*, one *mitis* 7 cases

PLATE XVII

FIG 4.—Tongue, pharynx, larynx, etc from a case of *gravis* diphtheria which died within three days of the onset of symptoms. Half of the left tonsil has been removed. Note the great enlargement and congestion of the tonsils, the inflammatory thickening of the pharyngeal wall, cedema of the ary epiglottic folds, and hemorrhagic enlargement of the cervical lymph glands. There is very little membrane formation and the larynx and trachea are devoid of macroscopical lesions. The larynx has been propped open by means of a glass rod.

FIG 5.—Part of a lung showing the naked eye appearance of the patchy hemorrhagic consolidation associated with the presence in the lung of both *C diphtheriae* and *H influenzae*.

and with the severity of the disease. As may be seen from the values of χ^2 and P the association is not one that would be likely to arise by chance. It is moreover of a relatively high degree, as the coefficient of association (Q_5) is approximately 0.9. It may be that this indicates that the action of *gravis* infection is particularly marked upon the lymphatic tissues generally, but such examinations as we have made of lymphatic glands other than cervical have not shown similar lesions. While hyperplasia and necrosis of the follicular reticulum cells of the spleen are of course by no means confined to diphtheria, these are remarkably constant and unusually well marked in the more severe cases of diphtheritic infection.

Liver.

Toxic changes were more marked in *gravis* and *intermedius* than in *mitis* cases. Well marked cloudy swelling was the usual finding. Fatty change was not conspicuous, and the fat which was present tended to be uniformly distributed in small droplets throughout the lobules. Many of the livers were oedematous. Multiple focal necroses have been previously recorded as a characteristic hepatic lesion, in our series these were observed three times, all in acute *gravis* cases. Swelling and some numerical increase of Kupffer cells were fairly constant findings. Nutmeg liver was seen in the congestive heart failure deaths.

Kidneys

In three of the present series the kidney changes were regarded as sufficient to justify the term nephritis. The first was a *mitis* case of 11 days' duration in which, in addition to severe degenerative changes in the tubules, there was conspicuous hyperplasia of the parietal epithelium of Bowman's capsule. The second was an *intermedius* case, also of 11 days' duration, in which in addition there was much hæmorrhage into the tubules. Focal embolic nephritis was encountered in an *intermedius* case, in which hæmolytic streptococcal septicæmia was also present. In several of the other cases changes were relatively more marked in the kidney than in any other viscus.

It is somewhat surprising that the most evident histological changes should have occurred in a *mitis* and an *intermedius* case, as in general the toxic changes in the kidney (nephrosis) were much greater in the acute *gravis* cases than in the others. These changes comprised marked swelling of the glomerular endothelium, with albuminous material in Bowman's space and occasionally adhesions between the tuft and the parietes, and extreme degrees of cloudy swelling (sometimes with associated fatty or hyaline droplet degeneration) of the tubules, and hyaline and granular casts in

Intermedius. 1 Hæmolytic streptococcal septicæmia with focal embolic nephritis

Mitis. 1 Rheumatic endocarditis and myocarditis Fibrosis and fusion of aortic and mitral cusps, acute dilatation, pericardial fluid about 400 ml, histologically the myocardium contained many characteristic Aschoff nodules

2 Patent interventricular septum The defect, about 1 cm in greatest diameter, was situated immediately below the aortic valve, and the septum for some distance around it was composed exclusively of fibrous tissue

3 Active caseous tuberculous focus in right lung, conglomerate tuberculoma of spleen, scattered miliary tubercles in liver

4 Chronic malnutrition in a young baby This is the exceptional *mitis* case previously referred to, in which the inflammatory process penetrated deeply into the tissue as in *gravis* cases It seems possible that the poor state of nutrition may explain the feeble resistance to infection

5 Acute tonsillitis *H influenza* recovered in large numbers from the tonsil The tonsils were quite unlike any others seen in the series, being enlarged, but pink on section and of fleshy consistency, histologically they showed superficial membrane of characteristic *mitis* type, but the lymphoid tissue was intact, though it contained numerous very large foci of reticulum cells

6 Death during the operation of tracheotomy The child died immediately after the knife entered the trachea At necropsy there was found between the œdematous ary-epiglottic folds a loose fragment of membrane which corresponded in shape and size to a denuded area on the tracheal surface at the incision The proximate cause of death would appear to have been spasm of the glottis

In addition to the above cases, there were three (one of each bacterial type) in which there was some evidence of an old myocarditis There were insufficient grounds, however, to justify us in forming any opinion as to its significance in relation to death

Causes of death

In many of our cases there were present at necropsy more than one lesion which might be regarded as the proximate cause of death, *e.g.* degenerative changes in the myocardium coincided in one case with an unusually extensive pneumonia We have attempted to classify the causes of death on as broad a basis as possible in table VIII, which serves to bring out one important point, namely that respiratory obstruction plays a more important part than general toxæmia in *mitis* cases, while the reverse is true for *gravis* and *intermedius* cases

A few points in amplification of table VIII should be mentioned Included under obstructive deaths there are not only those due to the membrane, but also the *mitis* case in which death occurred during tracheotomy (case 6 in the list given above) The tabulated toxic deaths include the 6 cases (two *gravis*, three *intermedius* and one *mitis*) in which myocarditis was the dominant lesion Of the remaining toxic deaths, the effects were most marked in the heart in 5 cases, in the kidney in 3 cases, in the suprarenal in 1 case and in the liver in 2 cases, in the others the changes were similar

time of its publication. The most complete account of the pathology of human diphtheria is probably that of Mallory (1908) and knowledge of the subject was summarised more recently by Andrewes *et al* (1923). In relation to the older work it will be sufficient to say that we have observed no individual lesions which have not been described already.

During the clinical course of *gravis* diphtheria it is usual to see in the fauces an amount of membrane exceeding the average for all diphtheria cases. It forms in the first place as a thin semi-translucent layer which may in the course of a few hours become firmer and changed in colour to grey, dark brown or black. When fragments separate they are unusually tough. The process is most marked over the greatly enlarged tonsils, but may also involve the faucial pillars, the posterior pharyngeal wall, the uvula and even the palate. This phenomenon has rarely been seen at necropsy and an explanation of the discrepancy between post-mortem findings and clinical observations on *gravis* infections is required.

In the first place it is clear from the difficulty which the diagnosis of *gravis* cases often presents to clinical observers in the early stages of the disease that marked membrane formation is not a feature of all cases and that in a certain number of the fulminant deaths there is no real discrepancy between the observations of clinician and pathologist.

There remain however numerous *gravis* cases in which clinical observation records marked formation of membrane both over the tonsil and passing beyond it to palate, uvula and even gums. Some of these recover and some die late, but there remain a number in which the absence of any marked membrane *post mortem* must be explained. Agonal or post-mortem proteolysis under the influence of cellular and bacterial ferments probably plays a considerable part and can readily be accepted as an explanation of the disappearance of the tonsillar membrane, since there the structure of the superficial membrane is practically indistinguishable from that of the exudate within the body of the tonsil. The extremely malodorous nature of the faucial contents strongly suggests the presence of a putrefactive process in many cases. It is not so easy to explain the disappearance of extratonsillar membrane, since we have little evidence of damage to the epithelium of the mouth in the immediate vicinity of the tonsil such as one would expect to find if a membrane involving the epithelium had recently been disintegrated. We doubt, however, whether the correlation of clinical and histological observations has been so meticulous in this series as to justify the statement that we have found an intact epithelium at death where shortly before a heavy membrane had been observed in the living patient.

The clinical infrequency of respiratory obstruction in *gravis*

muscle they were rarely found and then only as isolated organisms. The total number of demonstrable bacteria was not significantly different in *gravis*, *intermedius* and *mitis* cases.

It may be regarded as proved that the local defensive reactions are much more efficient in *mitis* infections than in the other types, and inasmuch as the predominant phenomenon in the local reaction is the formation of large amounts of fibrinous membrane, it is not surprising that the incidence of respiratory obstruction is highest in *mitis* cases.

The greater severity of the general toxic effects of *gravis* and *intermedius* infections than of *mitis* may possibly be attributable wholly or in part to the more effective local resistance in the latter. The general suggestion underlying our results as a whole is that the toxæmia differs quantitatively rather than qualitatively when the three types of infection are compared. The experimental protection of animals from infections with the different types of diphtheria bacilli, passively by the administration of antitoxin or actively with toxoid preparations, and the direct study of toxins obtained from these various types, have led to the conclusion that the toxins formed are identical, and in so far as the evidence of toxic action on the tissues of the body is concerned, our results would suggest a toxic action differing in degree rather than in quality. Thus although in the majority of instances the degenerative changes in the heart, liver, kidney etc. were more severe in *gravis* than in *mitis* cases, nevertheless the more severe manifestations of visceral involvement, e.g. myocarditis and nephritis, have also been seen in *mitis* cases. Moreover, in the exceptional *mitis* case already referred to the toxic changes in the viscera were of a profound order. The greater severity of the *gravis* and *intermedius* infections remains, however, to be explained. Assuming identity of toxins, the explanation might be either (a) that the *gravis* strain is the best producer of toxin *in vivo* notwithstanding its poor performance *in vitro*, or (b) that owing to its greater penetrating power, it produces toxin in sites from which the latter is more rapidly absorbed. Our post-mortem series does provide evidence for the second explanation, although it does not exclude the other possibility. It is still necessary to explain in some way the greater penetrating power of these strains. This may very possibly be due to toxic elements of which we have not yet got sufficient knowledge.

Tentative reference has been made by Cooper *et al.* to the limitation of hæmorrhages to *gravis* and *intermedius* cases and to the relatively greater frequency of these phenomena in the latter infections. In the present work we have regarded the post-mortem evidence of hæmorrhagic phenomena as unreliable. Such evidence is not as a rule striking. Subendocardial or subpericardial petechiæ may occur terminally in asphyxia and may not properly be refer-

No evidence of *C diphtheriae* bacillæmia has ever been obtained in this series

Focal necrosis of the malpighian bodies of the spleen is a significantly frequent concomitant of hæmorrhagic necrosis of the tonsil

While pneumonia in general does not seem to be an important cause of death, we have had occasional examples of a peculiar focal hæmorrhagic consolidation of the lungs, not obviously entirely inflammatory. In all such cases, both *C diphtheriae* and *H influenzae* were recovered from the pulmonary lesions

We have pleasure in expressing our appreciation of the facilities which have been afforded us by Dr E C Benn of Seacroft Hospital in carrying out this work and also in thanking Dr K E Cooper, who has helped with the bacteriological investigation of a few cases, and we are much indebted to Dr Matthew Young for help with the statistical analysis of numerical data. Reference has already been made in the text to the very generous assistance received from Professor H D Wright and Dr D T Robinson of Liverpool. We also wish to acknowledge with thanks a grant from the Medical Research Council in aid of expenses

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Microscopically the *gravis* colony is matt, flat, with crenated edge, the *mitis* smooth, reflecting the light, domed, with entire edge, and the *intermedius* matt or rough, small, flat or very obtusely conical, sometimes with slightly irregular edge

We have found that 2.5 per cent of agar is the best concentration but 1.5 per cent is satisfactory, 5 per cent is unsatisfactory both for growth and differentiation

Meat extract is necessary for good differentiation and an extract made from frozen ox heart is generally better than one made from fresh veal

The appearance of the colonies is apt to vary a little with different batches of nutrient agar, even though these are made by the same person and contain the same percentage of blood of the one animal. It is, however, easy to distinguish with the naked eye between the three types of colony on any batch of nutrient agar containing 10 per cent of guinea-pig or rabbit blood or on good quality nutrient agar containing 5 or 2.5 per cent of these bloods. Differentiation is difficult on good agar containing 10 per cent of sheep blood and is practically impossible with 5 per cent of sheep blood or with human or horse blood

The colony microscope is hardly necessary with 10 per cent of guinea-pig or rabbit blood on any batch of agar or with 5 or 2.5 per cent guinea-pig or rabbit blood on good agar. With 10 per cent of sheep blood, unless the agar is of the best quality, the microscope is indispensable for distinguishing between *gravis* and *mitis* colonies, those of *intermedius* type are easily recognisable with the unaided eye

Because guinea-pig blood agar was so satisfactory, it was thought that Clauberg's medium might be improved by substituting guinea-pig blood for sheep blood. But Clauberg's medium is practically useless when made with guinea-pig blood, which is not completely lysed by glycerin. The medium is therefore opaque instead of transparent and growth of *C. diphtheriae* is sparse and type differentiation difficult before 48 hours

Discussion

Of the many reports since that of Anderson, Happold, McLeod and Thomson (1931) it is remarkable that there are apparently only two which mention the characters of the various types of *C. diphtheriae* colonies on blood-agar. Preuss (1936) reported that the types can be differentiated on human or rabbit blood agar and Puschel (1936-37) that the colonies of the three types can be distinguished readily on guinea-pig blood agar*. Puschel's description agrees with ours. She recommended freshly poured plates but we have found them satisfactory up to one week.

Clauberg's (1931) medium is excellent for recognising and

* Similar results are recorded by Gundel and Tietz (1934-35) —Ed

The quality of the nutrient agar influences differentiation less with guinea-pig or rabbit blood than with sheep blood, with which some batches of nutrient agar are not satisfactory. With 10 per cent of guinea-pig blood differentiation is excellent on any of the nutrient agar bases tried.

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organisms other than sarcinæ, diphtheroids and diphtheria bacilli in the cultures. Diphtheroids can in most instances be readily distinguished by their shortness and tendency to divide evenly. Diphtheria bacilli are longer, especially those of the *mitis* type, may be barred, especially those of *intermedius* type, or if shorter, may be pointed at one end and show a tendency to divide into two portions of unequal length, seen especially in the *gravis* type. Pleomorphism is sometimes well marked especially with certain *mitis* strains. The growth on tellurite blood agar is somewhat slower than on Loeffler's medium and we have found that it is advisable to reserve it for swabs arriving at the laboratory in time to allow of a full 16 hours' incubation before examination.

The accuracy of identification of diphtheria bacilli in this way may be judged by the fact that of 553 throat swabs which were considered positive, 541 were confirmed after isolation and of 255 nose swabs 248 were similarly confirmed. In all, of 808 microscopic diagnoses from films of growth on tellurite blood agar, 789 (97.7 per cent) were confirmed while 19 (2.3 per cent) were false. These results were obtained after a considerable experience of the method. The chief difficulty has lain in distinguishing barred diphtheroids from diphtheria bacilli of the *intermedius* type and certain, usually *gravis*, strains from diphtheroids of similar size. Granulation of the bacilli is usually poorly developed.

The microscopic diagnosis was reported and all positive results were checked by plating on tellurite blood agar. Where the microscopic result was negative the culture on the Loeffler slope was emulsified in saline and a portion plated on tellurite blood agar. Further swabs from negative cases were examined on each of the two following days unless the second examination gave a positive result.

Altogether 1684 cases were examined in this way, of which 1129 were finally diagnosed as diphtheria and 555 were found to be suffering from some other disease. All had been notified as diphtheria prior to admission. The microscopic diagnosis is that arrived at after examination of films from both the cultures.

In the cases which clinically were not diphtheria the microscopic findings were as follows

	Cases	Per cent
No <i>C. diphtheriæ</i> seen in films or isolated on plates	526	94.8
<i>C. diphtheriæ</i> seen but on isolation proved non-virulent	14	2.5
<i>C. diphtheriæ</i> not seen in films but isolated on plates and proved non-virulent	2	0.4
<i>C. diphtheriæ</i> thought to be present in films but not isolated (false-+)	13	2.3
	<hr/> 555	

The error of the microscopic method as compared with the clinical diagnosis was in this group a little over 5 per cent—2.9 per cent due to the presence of non-virulent organisms of *mitis*

unnecessary to make repeated diagnostic swabbings if the results accord with the clinical opinion but that this is valuable where any discrepancies occur

Accuracy of microscopic identification of diphtheria bacilli

We have used the material available to estimate the accuracy of a microscopic identification of the diphtheria bacillus based on the examination of cultures on both Loeffler's medium and tellurite blood agar slopes as judged by subsequent isolation on tellurite blood agar plates. Our information relates to the examination of 1675 swabs (table II)

TABLE II

Correctness of microscopic identification as judged by plating

Category	Swabs examined	Microscopic — Plate —	+	+	—
			+	—	+
Diphtheria cases	748	57	670	0	21
Cases not diphtheria	927	885	22	16	4
Total	1675	942	692	16	25

Judged by this standard the microscopic identification was correct with 1644 swabs (97·5 per cent), yielded false positives with 16 (1 per cent) and false negatives in 25 (1·5 per cent), the total error being about 2·5 per cent

Value of nasal swabs for diphtheria diagnosis

As we wished to simplify our method without sacrificing the advantages of using two media and employing plates we decided to limit the examination of nasal swabs as far as possible. This appears to be justified by the fact that we have compared the results of examining 839 pairs of swabs from cases of diphtheria and found that 565 (67·3 per cent) were positive in the throat and negative in the nose, 257 (30·6 per cent) were positive in both, while 16 were positive in the nose and negative in the throat. One case was negative in both but a positive culture was obtained from the trachea. In only 16 cases therefore of those examined for diagnostic purposes was any advantage found in examining nasal swabs. These cases were all suffering from either nasal or laryngeal diphtheria. In one case of the latter an *intermedius* strain was isolated from the trachea and nose and a non-virulent *mitis* strain from the throat. In our later work we therefore limited our examination of nasal swabs to cases with nasal discharge and laryngeal cases. This limitation applies only to the diagnosis of diphtheria and not to cases examined prior to discharge from

checking of our microscopic methods was undertaken, an experience not without profit to those concerned. Possibly certain advantages are attached to the "wet" method of staining. We do not think that the relatively favourable results with Loeffler's medium was due to a general inefficiency in our methods. During the period 1571 cases of diphtheria were diagnosed clinically. In 1554 (98·9 per cent) the swabs were positive and in 17 (1·1 per cent) negative. In our experience the combination of the Loeffler slope and the tellurite blood agar plate gives the maximum of accuracy with the minimum of work. The virtue of the introduction of the tellurite plate seems to us to lie not so much in its greater efficiency, though we think it is more efficient on the whole, as in the check it provides on the microscopic work and the replacement of sometimes dogmatic opinion by ascertained fact. The double method is well worth the extra work because of the added sense of security which it gives. Even with it we are still left with a discrepancy of about 1 per cent. between clinical opinion and laboratory findings, a discrepancy which so far we have not been able to eliminate.

We have not had extensive experience of Clauberg's medium, the latest prescription for which is given in detail by Sutherland and Iredale, but we have made some observations on its use. The agar basis we have used is the ordinary one currently employed in these laboratories made from beef and proteose peptone and appears to be satisfactory. In general we have found that *gravis* and *intermedius* strains grow well and produce good colouration in 24 hours but a proportion of *mitis* strains grow poorly and produce little or no colour in that time though this might appear later. On the other hand many cocci produce a blue colour and certain diphtheroids produce large amounts of alkali and may prevent the development of an acid reaction by diphtheria bacilli in their neighbourhood. We think the medium complicated and have endeavoured to simplify it. Our experiments indicate that the blue colour depends on the production of sufficient acid to change the colour of the slowly reacting water blue. For this the glucose is essential but the glycerol plays no part in the change. The sodium acetate can apparently be omitted without disadvantage. Cystin however is essential, as it permits of heavier growth and better acid production. The lysed blood cannot be safely reduced much below 16 per cent but either sheep or ox blood may be used and we have found that Seitz-filtered lysed sheep blood is entirely satisfactory. We have been unable to satisfy ourselves that the addition of glycerinated blood which has been allowed to "ripen" for six weeks adds anything to the value of the medium. A simply prepared medium consisting of beef infusion agar, sheep or ox blood (16 per cent), potassium tellurite (0·4 per cent), glucose (1·5 per cent), cystin and the dye solutions made up and used as described by Sutherland and Iredale has given us as good results as the more complicated medium. It serves as a good check on any microscopic method used, but in a small series of cases examined prior to discharge from hospital gave results which were rather inferior to those obtained by microscopic examination of films from cultures on tellurite blood agar slopes. We have not persisted with the medium, as it is quite useless for typing the organisms and this was one of the main objects of our investigation.

20 were aged 5-9, 9 were 10-14 and 18 were 15 or over. It is in the age group 15+ that we have met with most difficulty. It is in this period that the notification error is greatest and we have found that many cases with the clinical features of diphtheria are in fact cases of streptococcal tonsillitis. Of late we have regularly examined swabs from such cases for hæmolytic streptococci. Just how many of the 59 patients were really suffering from diphtheria it is impossible to decide. Some certainly were, for two subsequently developed mild paralysis and one or two had mild circulatory disorders. Some difficulty has also been experienced with laryngeal cases and in one or two instances we have been able to isolate diphtheria bacilli only from a tracheotomy wound or from the nose. We are satisfied that repeated nasal and throat swabs should be examined from all such cases. On the other hand we have met with at least three cases requiring tracheotomy which were not diphtheritic.

We think it evident that it is well worth while to confirm all diagnoses of diphtheria bacteriologically and in the absence of such confirmation to review the diagnosis, not so much for purposes of treatment as for administrative reasons, for the patient who is not suffering from diphtheria should not be exposed to the risks of detention in a diphtheria ward in which cross infection is rife and may lead to serious illness or prolonged hospitalisation.

The error of notification of diphtheria at various age periods

In estimating the severity of diphtheria in a community the case fatality rate is the only standard available. It is probable that the error of notification of deaths from diphtheria is not large and therefore the accuracy of notification of cases is of great importance. It is generally recognised that this is liable to considerable error and that case fatality rates must be accepted with reserve. We find that the crude rate for our series is in error by approximately 40 per cent for cases at all ages. The error at different age periods is recorded in table V.

TABLE V

The error of notification of diphtheria at various age periods

Category	Age period					Total (age known)
	Not known	0-4	5-9	10-14	15 +	
Diphtheria *	35	822	1375	405	218	2820
Not diphtheria	24	409	486	366	512	1773
Notified as diphtheria	59	1231	1861	771	730	4593
Percentage not diphtheria		33.2	26.1	47.5	70.1	38.6

* Confirmed bacteriologically, carriers excluded

We are indebted to the Medical Officer of Health of Liverpool, Professor W M Frazer, and his assistants for many facilities afforded in this investigation, to Dr A E Hodgson for a great deal of assistance with the clinical material and especially to Dr F Weightman, Dr A B Concanon and other resident medical officers for much clinical help without which our study would have been impossible

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opaque greyish growth with a rather dry surface and slightly irregular edge. All *mitis* strains were examined for their power to ferment glucose, sucrose and starch, to distinguish them from diphtheroids on the one hand and certain *gravis* strains on the other. *Intermedius* strains were examined only for power to ferment sucrose, as the only difficulty here arose from confusion with a sucrose-fermenting diphtheroid, and *gravis* strains for their action on starch.

This technique appears to us to be the minimum for type differentiation but it has sufficed in most instances. Doubtful strains have been examined more fully and we are indebted to Dr D T Robinson for examining a large number serologically. All the *gravis* strains except 3 fell into his type I (Robinson and Peeney, 1936) and these 3 were all of type III, showed coccoid morphology and produced smooth colonies. All the *intermedius* strains tested fell into one serological group but the *mitis* strains were heterogeneous. Atypical strains were very uncommon, nine strains in all, they produced colonies of the *gravis* type but failed to ferment starch and were virulent. Six of them came from one district of Liverpool and from neighbouring streets. Our experience has been that if we had relied on colony formation as the only criterion the error would have been quite small and the additional tests have been sufficient to obviate this. The technique has seemed to us to be relatively simple but to require a little experience. Virulence tests were done on *mitis* strains from doubtful cases or from carriers but not on those from frank cases of diphtheria or on *intermedius* or *gravis* strains.

Incidence of the various types

This is shown in table I

TABLE I

Incidence of types of diphtheria bacilli in cases of diphtheria

Group	<i>Mitis</i>		<i>Intermedius</i>		<i>Gravis</i>		Total
	Number	Per cent	Number	Per cent	Number	Per cent	
1936 A	175	25.9	228	33.7	273	40.4	676
1937 A	608	42.6	343	24.0	476	33.4	1427
1937 B	288	38.3	195	25.9	269	35.8	752
Total cases	1071	37.5	766	26.8	1018	35.7	2855

The figures indicate that all three types are well represented in Liverpool and suggest some fluctuation in frequency. The figures for the individual quarters of the year are given in table II. It should be appreciated that those for 1936 refer only to a proportion of the total cases in the city, whereas for 1937 they are relatively complete.

On the whole the incidence of the *gravis* type has been fairly constant, whereas the *mitis* type tended to supplant the *intermedius* in the summer of 1937. The fluctuations are less marked than those quoted by Robinson and Marshall (1935) for Manchester, but it may well be valuable to follow these changes in future years in

There are only three districts in which the excess representation of any one type appears to be statistically significant. In Edge Hill the *mitis* type accounts for 52.5 per cent (difference from city average (D) = 11 per cent, standard error of difference (S E) = 3.6), in Wavertree the *intermedius* type for 45.8 per cent (D = 21.4, S E = 3.5) and in Toxteth the *gravis* type for 51.0 per cent (D = 17, S E 3.25).

The figures obtained for carriers are perhaps not of much significance as there appears to be considerable variation of opinion as to what constitutes a carrier. There were in all 244 patients diagnosed as such and in 109 of these (44.7 per cent.) the organism was of the *mitis* type, in 46 (18.9 per cent.) of the *intermedius*, in 85 (34.8 per cent.) of the *gravis*. Mixed types were found in 4 cases (1.6 per cent.) These figures refer to the whole period of the enquiry and we do not know how far they represent the state of affairs in the general population.

Age incidence of infection with the various types
Our figures relating to this are given in table IV.

TABLE IV
Incidence of types in different age groups

Type		Age group					Total (age known)
		Not known	0-4	5-9	10-14	15+	
<i>Mitis</i>	Number	16	335	515	130	75	1055
	Per cent *		31.8	48.8	12.3	7.1	
<i>Intermedius</i>	Number	14	220	345	133	54	752
	Per cent		29.3	45.9	17.7	7.2	
<i>Gravis</i>	Number	5	267	515	142	89	1013
	Per cent		26.4	50.8	14.0	8.8	
All types	Number	35	822	1375	405	218	2820
	Per cent		29.1	48.8	14.4	7.7	

* Percentage of total cases whose age is known falling within the age group

These figures suggest that a slightly greater proportion of the *mitis* cases occur in the age period 0-4 years and of the other types in the later periods, but the differences are small and in only one instance (*intermedius-mitis* at age 10-14) equal to three times the standard error. It is of some interest that the proportion of all cases which falls in the age period 15+ is only 7.7. We have pointed out elsewhere (Shone *et al.*, 1939) that it is in this age period that the greatest error of case notification is to be found, and in most of the reports of Medical Officers of Health of cities which we have studied the proportion of cases notified which falls

rate but this is significantly less than for the *intermedius* (D/S E = 6.8) and *gravis* cases (D/S E = 4.0). The rate for the *intermedius* cases is also significantly higher than that for the *gravis* (D/S E = 3.0). The feature of the series is that the *intermedius* cases are at least as severe as the *gravis* and in some respects even more serious. Both are more severe than the *mitis* cases, though these are by no means negligible.

TABLE VI

Frequency of complications in infections with different types

Type	No. of cases	Cases with complications	
		Number	Per cent
<i>Mitis</i>	783	124	15.8
<i>Intermedius</i>	571	180	31.5
<i>Gravis</i>	749	180	24.0
All types	2103	484	23.0

Frequency of individual complications

In estimating this, each complication has been considered separately and any case with more than one complication appears in more than one category (table VII).

TABLE VII

Frequency of individual complications with different types

Type	No. of Cases	Complications											
		Laryngeal		Tracheotomy		Circulatory		Paralysis		Hæmorrhage		Severe toxæmia	
		No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent
<i>Mitis</i>	783	68	8.7	41	5.2	29	3.7	22	2.8	2	0.3	2	0.3
<i>Intermedius</i>	571	5	0.9	3	0.5	83	14.5	90	15.8	8	1.4	12	2.1
<i>Gravis</i>	749	7	0.9	6	0.8	82	10.9	92	12.3	4	0.5	12	1.6
All types	2103	80	3.8	50	2.4	194	9.2	204	9.7	14	0.7	26	1.2

In compiling this table no attempt has been made to separate primary from secondary laryngeal involvement, to distinguish grades of severity in the complications or to separate cardiac from other circulatory involvement. The severe toxæmias were those without any specially localised effect. Fatal cases have been included but adenitis and otitis media have not been considered.

the type to be the same as he has met with in all parts of England and Scotland

The causes of death in infections with the three types as judged by the clinicians concerned are given in table IX

TABLE IX

Causes of death in infections with different types

Type	Deaths	Cause of death									
		Laryngeal obstruction		Early toxic				Late toxic		Pneumonia	
				1st week		2-3 weeks		5 weeks +			
		No	Per cent *	No	Per cent *	No	Per cent *	No	Per cent *	No	Per cent *
<i>Mitis</i>	26	9	34.6	4	15.4	9	34.6	1	3.9	3	11.5
<i>Intermedius</i>	82	1	1.2	30	36.6	38	46.3	11	13.4	2	2.4
<i>Gravis</i>	67	3	4.5	25	37.3	27	40.3	12	17.9	0	
All types	175	13	7.4	59	33.7	74	42.3	24	13.7	5	2.9

* Percentage of total deaths due to a given type which fall into this category

Of the deaths in *mitis* cases 9 were due to laryngeal obstruction and 5 others occurred in patients who had undergone a tracheotomy, though in these the death was considered to be due to toxæmia. Two *intermedius* and 4 *gravis* cases had also undergone tracheotomy. It is clear that a *mitis* infection is by no means negligible but the main risk arises from laryngeal and pneumonic involvement. In the other two groups toxæmia is responsible for more than 95 per cent of the deaths, the proportions being very similar in both series. For the *intermedius* and *mitis* deaths the incidence of toxæmia as the main cause differs by 42.6 per cent, which is 5.5 times its standard error.

Case fatality rate of different types at different age periods

In table X we summarise our findings on this point.

These findings are compared and analysed in table XI.

We have considered as statistically significant only those differences which are equal to at least three times their standard error. On this basis it is seen that the *mitis* case fatality rate is significantly lower than that for the *intermedius* cases in the age groups 0-4 and 5-9, while the difference is also highly suggestive of significance in the group 10-14. The *gravis* rate is not significantly higher than the *mitis* rate in the 0-4 group but is in the 5-9 group it is significantly lower than the *intermedius* rate in the 0-4 group.

but not in the others. The *intermedius* rate is significantly higher than the *mitis* in the first two periods and probably also in the third, it is significantly higher than the *gravis* rate only in the age period 0-4. The fatality rates for the individual types at different age periods are compared in table XII.

TABLE XII

Comparison of case fatality rates of each type at different age periods

Pairs	Type								
	<i>Mitis</i>			<i>Intermedius</i>			<i>Gravis</i>		
	D	SE	D/SE	D	SE	D/SE	D	SE	D/SE
0-4 and 5-9	+5.5	1.2	4.6	+9.2	2.3	4.0	+1.0	2.1	0.5
0-4, 10-14	+5.5	2.2	2.5	+10.7	4.0	2.7	+5.8	2.6	2.2
5-9, 10-14	0			+1.5	2.9	0.5	+4.8	2.1	2.3

The case fatality rate is significantly higher at 0-4 than at 5-9 for both the *mitis* and *intermedius* types, it is also higher but not quite certainly significantly higher in this period than at 10-14. The doubt is probably due to the small numbers in the third group. The differences are quite insignificant for both types in the 5-9 and 10-14 groups. For the *gravis* cases the difference in the rates for the 0-4 and 5-9 age periods is remarkably small and quite insignificant. In the 10-14 period the rate is lower but the difference is not certainly of significance. For all cases without distinction of type the fatality rate at 0-4 years is significantly higher than at 5-9 or 10-14 ($D/SE = 4.4$ and 3.8 respectively) but there is no significant difference between the rates in the two higher age groups ($D/SE = 1.4$).

In tables X and XI are also given the proportion of deaths due to each of the types which occurred in the given age groups. From table XI it will be seen that the proportion of *mitis* deaths in the 0-4 period is significantly higher than the proportion of *gravis* deaths and almost certainly significantly higher than that of the *intermedius* deaths. In the second age period (5-9) the percentage of *mitis* deaths is significantly lower than that of *gravis* but the difference is not quite certainly significant as between *mitis* and *intermedius* or *intermedius* and *gravis*. In the 10-14 period none of the differences are great enough to be of importance.

On the basis of this analysis it would appear that in a pure epidemic of diphtheria due to organisms of the *mitis* type under the conditions prevailing at present in Liverpool the age distribution of the cases would be very similar to that in an epidemic due to

later in the disease. If anything cases in these groups, as Robinson and Marshall (1935) found in Manchester, tend to be admitted earlier. Cases may in fact be admitted late because they are mild and not necessarily because they have been neglected.

The information relating to the fatal cases is contained in table XIV.

TABLE XIV
Day of admission of fatal cases

Type	Day of admission							Total no in which day of admission known
	Not known	1	2	3	4	5	5+	
<i>Mitis</i>	1	0	4	4	11	2	4	25
		8 = 32 per cent			17 = 68 per cent			
<i>Intermedius</i>	1	1	12	29	18	13	8	81
		42 = 52 per cent			39 = 48 per cent			
<i>Gravis</i>	0	1	8	20	26	7	5	67
		29 = 43 per cent			38 = 57 per cent			
All types	2	2	24	53	55	22	17	173

Of the fatal *mitis* cases 8 (32 per cent), of the *intermedius* 42 (52 per cent) and of the *gravis* 29 (43 per cent.) were admitted before the end of the 3rd day of disease. The differences are not certainly significant but so far as they go they indicate that the *intermedius* and *gravis* cases were not admitted especially late, rather the reverse. Such delay as there was in admitting these fatal cases to hospital was not largely due to waiting for a laboratory report, for of the 173 fatal cases only 20 were bacteriologically examined before admission to hospital.

Serum treatment

Our information as to serum treatment is limited to the dosage given to 145 fatal cases without reference to the weight of the patient or stage of the disease. Two patients only, both *mitis* infections who died almost immediately after admission, received no serum. The details are summarised in table XV.

There is no evidence that the dosage is greater in the *mitis* than in the other cases and this may mean that not only are the *intermedius* and *gravis* cases recognised a little earlier but also that they are generally recognised as more severe and given larger doses of serum. It must be remembered however that the *mitis* deaths tend to occur in a younger age group and so would include smaller children and this might result in smaller doses being given.

All the evidence together seems to us to indicate that the differences in severity which have been observed are really the result

previously but not Schick tested Her condition was precisely similar to that of her sister and she died within 12 hours of admission

Severity of diphtheria in different localities.

The view originally put forward by the Leeds workers (Anderson *et al*) that the severity of diphtheria depended in part upon the type of organism present, though at first disputed, has since received adequate confirmation The precise degree of severity of the infections associated with the different types has seemed to vary somewhat from place to place and perhaps from time to time Cooper *et al* (1935-36) have reported figures from a number of sources which bring out these variations but do not indicate their statistical significance We have therefore recalculated and rearranged these figures, omitting the London ones as the method of type determination has been somewhat different (Mair, 1936), and including all the Leeds figures and not simply the smaller group in table V of the paper referred to and have added our own figures for Liverpool (table XVI)

TABLE XVI

Case fatality rate of diphtheria for different types in different cities

Place	Date	Mitis			Intermedius			Gravis		
		Cases	Deaths	Case fatality rate	Cases	Deaths	Case fatality rate	Cases	Deaths	Case fatality rate
Cork	1934-35	3	0		3	0		74	13	17.6
Berlin	1934-35	*34	0		32	3	9.4	287	38	13.2
Manchester	1933-34	274	3	1.1	449	57	12.7	552	82	14.9
Hull	1932-33	*37	1	2.7	141	7	4.9	222	37	16.7
Dundee	1933-34	59	2	3.4	90	10	11.1	9	0	
Leeds (1) †	1931-32	177	3	1.7	54	2	3.7	485	54	11.1
	and 1935 *									
Liverpool	1936-37	1071	26	2.4	766	82	10.7	1018	67	6.6
Leeds (2) ‡	1931-35	*355	5	1.4	286	14	4.9	2115	133	6.3
Durham and Newcastle	1933-34	88	5	5.6	73	4	5.5	30	2	5.1
Glasgow	1932-33	*125	9	7.2	346	17	4.9	14	1	7.1
Edinburgh	1932-35	112	4	3.6	208	12	5.8	9	0	
Stafford	1932-34	622	9	1.4	118	5	4.2	73	4	5.5
Total		2780	64	2.3	2512	211	8.4	4412	377	8.5

* Atypical cases excluded

† A special group—not included in totals

‡ All cases typed, including Leeds (1)

A comparison of the differences in fatality rates for the different areas is given in table XVII

In this table it can be seen that many of the differences, even though large, are not certainly significant It appears that the case fatality rate for *gravis* infections is significantly larger than

Summary

1 The incidence of the various types of diphtheria bacilli in 2960 cases of diphtheria in Liverpool is reported

2 There is some evidence of seasonal variation, a little of a tendency for localisation of certain types in certain districts but practically none of selective incidence in age groups

3 The severity and case fatality rate have been greatest with the *intermedius* type and least with the *mitis*.

4. Toxic complications have been most frequent with the *gravis* and *intermedius* types, laryngeal and pneumonic with the *mitis*

5. The case fatality rate in *mitis* cases is much higher in children under 5 years of age than in older ones, in *intermedius* cases the rates are higher at all age periods and especially so in the older children, in *gravis* cases the rate at 0-4 years is very similar to that at 5-9 years

6 The observed differences between the different types of infection do not appear to depend on differences in the time of admission to hospital or of serum treatment

7 The findings of other observers are compared and discussed

We are indebted to the Medical Officer of Health for Liverpool (Professor W M Frazer) and his staff for the opportunity of making these observations and to Dr A E Hodgson and the staff of the Fazakerley City Hospitals for much clinical assistance. We are particularly indebted to Dr F Weightman and Dr P Unsworth for invaluable clinical help in the early stages of the enquiry and to them and to Drs A B Concanon and F Dodd and other resident medical officers for similar assistance later

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slopes, as Braun (1916) found, belated growth of diphtheria bacilli or tardy formation of granules was occasionally noticed. In 2 cases the findings were positive on Loeffler slopes while they remained negative on Gundel-Tietz plates even after 48 hours. The organisms from these slopes yielded typical diphtheria colonies on the tellurite plates and gave correct fermentation of sugars. As all swabs were first smeared on Loeffler slopes and then on plates, the failure of growth on tellurite plates does not prove the failure of the method in either case.

The macroscopic diagnosis of diphtheria by direct examination of Gundel-Tietz plates gave over twice as many positive results as the microscopic (Loeffler tube) method. We consider that the most important cause of this discrepancy was the fact that 72 per cent of our cases were *gravis* infections and the *gravis* bacilli, especially during the severest phase of the epidemic, deviated very much in their morphology from the classical form. They were very short and most of them had no granules or only one. This atypical morphology will necessarily lead to a number of mistakes where microscopic diagnosis only is used.

Early often we found a sucrose-fermenting diphtheroid whose morphology is very like that of the diphtheria bacillus (Hettche, 1935, Breuner, 1936a). On agar, Loeffler and heated blood agar it forms a moist, whitish and abundant growth. On tellurite plates the colonies are remarkable for their size and for the fact that in the heavily inoculated areas they are black, while single colonies are transparent or opaque, greyish white and very shining. Genuine diphtheria cultures on the contrary show a lighter shade where the colonies are confluent, while single colonies are dark or black. These strains ferment both glucose and sucrose and so they form blue colonies on Clauberg's indicator medium III (1935). We found them in 3 per cent of our cases. They were isolated mainly from the nose and ears and from wounds. As this organism is frequently found and can easily be mistaken for *C. diphtheriae*, we prefer the Gundel-Tietz medium to Clauberg III. This *Corynebacterium* seems to be identical with the paradiphtheria bacillus of Lubinski (1920-21) which Hettche (1935-36) rediscovered and called hyperacid pseudodiphtheria. The strains are non-pathogenic. They grow anaerobically but aerobic growth is far better.

Distribution of types

Our material came from two different groups of patients. The first and most important group was drawn from cases in the 8th Soviet Children's Hospital for Infectious Diseases in Kharkoff. The swabs of this group were taken at the same time from throat and nose, but examined separately. The second group came from

Pesch believes in the possibility of a change of type, while Hettche (1935), Preuner (1936b) and Weigmann and Koehn (1936) consider the change of one type into another and also into diphtheroids as possible

In this connection 33 cases were investigated several times weekly during the whole period of stay in hospital this varied between a fortnight and three months With the exception of one case, which at the first examination yielded atypical and later *gravis* bacilli, the original type persisted throughout the whole course of the illness In 7 cases, as mentioned before, in addition to the original type a small number of colonies of another type were found which may have come from a secondary infection

These results cannot be regarded as a proof that the types are constant Better evidence for that conclusion is provided by the observation that in multiple cases occurring in one family the type was always uniform We had 7 instances of such family infections, 5 with two patients each and 2 with three patients In four families we found only *mitis* strains and in three only *gravis*.

Laboratory investigations prove more definitely the constancy of types. Three *gravis*, 3 *mitis* and 2 *intermedius* strains were subcultured 50 times on tellurite medium every 3 days They were also kept on broth for 6 months, being subcultured once every two months All three types remained unchanged except that two of the *gravis* strains grew in broth with uniform turbidity and no pellicle Growth in broth does not seem to us to be as satisfactory a differential test as sugar fermentation We also passed one *gravis* and one *mitis* strain 30 times through guinea-pigs without finding any changes in their fermentative activities or their pathogenicity

Relation of type of infection to clinical severity

In their first publications Anderson *et al* found a close relationship between the type of organism and the severity and character of the disease This was questioned by Parish, Whatley and O'Brien (1932), Wright and Rankin (1932), Menton, Cooper, Duke and Fussell (1933) and Mair (1936) but later it was generally accepted In Germany the reverse has happened The first publications agreed with the Leeds findings (Christison, 1934-35, Schiff and Werber, 1935, Hettche, 1935, Gundel and Liebetrueth, 1935-36, Clauberg, Helmreich and Vierthaler, 1936) and only recently has there been an increase in publications which state that no such relation exists (Mittag and Otto, 1936, Hilgers and Thoenes, 1936, von Bormann, 1937, Becker, 1937) The first investigations in Soviet Russia (Shapiro and Rukevitch, 1937, Zdrodowski and Halapine, 1936) did not demonstrate any relationship between type and illness However, during the time in which they collected materials for their work the Health Offices in Moscow reported only endemic cases of diphtheria The relationship between type and clinical severity is not usually so pronounced in endemic cases

We were able to make use of the histories of all cases from the Diphtheria Department of the 8th Soviet Children's Hospital

years 1934-35, with alum-precipitated anatoxin prepared in the Toxin Department of the Ukrainian Metchnikoff Institute. When the epidemic broke out, an attempt was made in several Children's Homes to assess the effectiveness of the previous immunisation. This investigation revealed that 40 per cent. of the "immunised" children were Schick-positive. Therefore it cannot be denied that either the method of one-shot immunisation or the particular preparation of anatoxin used was inadequate in many cases.

Despite this state of affairs it seemed of some interest to determine how many of those who fell ill had been immunised, how the illness evolved in such individuals and what proportion of the fatal cases were included in those who had been immunised. The 254 hospital cases could be divided into three groups from the point of view of immunisation: (a) those actively immunised (83), (b) those from whom no clear account of immunisation could be obtained (68) and (c) those definitely not immunised (103). The distribution of the types within these three groups did not present any interesting features except that the *gravis* type occurred in the immunised group with 12 per cent. greater frequency than in the non-immunised group. In respect of the number of severe, moderate and slight cases, all three groups were roughly similar. The deaths in group (a) numbered 4 (4.8 per cent.), in group (b) 10 (14.7 per cent.) and in group (c) 11 (10.7 per cent.). No conclusion can be drawn from these observations as the differences are not statistically significant.

Observations in dispensary cases

The *gravis* and *mitis* infections amongst the dispensary cases were not in the same proportion as in the hospital group. This was apparently due to the method of sending material for culture and to the type of cases available. Among 228 cases the *mitis* type occurred 125 times, the *gravis* 102. In one case the organism defied classification and was considered an atypical strain. No case of *intermedius* infection was observed. It was remarkable that in this group the differentiation of types by the appearance of growth in primary culture was often difficult and occasionally impossible, and growth in broth was often irregular.

VIRULENCE AND POWERS OF INVASION OF THE TYPES OF DIPHTHERIA BACILLI

The bacteriological and clinical findings during the Khaikoff epidemic showed a correspondence between clinical severity and bacterial type which accorded with the experience of the Leeds workers. In view of these facts we proceeded to determine whether the greater pathogenicity of the *gravis* strains could be corroborated by animal experiment.

guinea-pigs injected with *gravis* strains was 29.4 hours, while for those injected with virulent *mitis* strains it was 54.2 hours. Thus in animal experiments the Kharkoff *gravis* strains were more virulent than the *mitis*.

Generalisation of infection in guinea-pigs.

Gundel and Erzin (1935) and Gins (1935-36) suggest that with a better technique it would be possible to obtain growth of diphtheria bacilli from the internal organs of infected guinea-pigs. As a result of their experiments Gundel and Erzin attributed a greater power of invasion to the *gravis* type. Clauberg (1936) objected that the subcutaneous method of injection afforded opportunity to the diphtheria bacilli to find their way into the circulating blood on account of the damage inflicted on the smallest blood vessels. For this reason he thought such observations were not convincing and also he noted that bacteria were not found in the organs after infection of the vaginal mucous membrane of guinea-pigs. In a later publication (Clauberg and Plenge, 1937) he came to the conclusion, as a result of the examination of organs in 23 autopsies, that a diphtheria bacillæmia may occur, while Kroemer (1936-37) found diphtheria bacilli in the organs of 10 cases *post mortem*, but as a result of his histological examinations he considers that this is of no importance in determining the course of the illness. Isabolinski and Karpatschewskaja (1932), Chiari (1935) and Ciantini (1936) also arrive at the conclusion that in diphtheria bacillæmia is not as rare as was formerly believed.

As a contribution to this question, we made inoculations on tellurite plates, as soon as possible after death, from the organs of guinea-pigs used in the virulence experiments. Investigations were made of the pleural fluid, lungs, heart blood, heart muscle, peritoneal fluid, liver, spleen, kidneys, suprarenals and tonsils and of the inflammatory zone at the site of infection. The tellurite plates were inoculated according to the technique described in the first part of this paper for the examination of human tissues *post mortem*. Amongst the 45 animals which died of *gravis* infection there were 18 (40 per cent) in which bacilli identical with those injected were recovered from the viscera and the serous exudate and 44 in which they were recovered from the tissues at the site of injection. None were ever recovered from the heart blood. Most frequently the bacilli were found in the liver, spleen and peritoneal fluid, most rarely in the kidneys and the tonsils. In one case only *gravis* organisms were recovered from the tonsils and in this case only 2 colonies appeared. Quite often, however, we found in the tonsils glucose-fermenting diphtheroids without pathogenic significance. They could only be differentiated from genuine diphtheria bacilli by saccharose fermentation, growth in deep glucose agar and lack of virulence. The same organisms were isolated from the tonsils of 20 control guinea-pigs which had died of anaphylactic shock. Thus no support was given to the assumption made by Gundel and Erzin (1935) that diphtheria bacilli

Experiment 1. In a preliminary investigation 20 guinea-pigs of 250 g each received a single dose of 5 c c. of anatoxin from the strain Park-Williams 8. One month later these animals together with one non-immunised control each received 30 M L D of *gravis* toxin. The control died in 48 hours and showed typical post-mortem changes. Four of the immunised animals showed slight infiltration at the site of injection, but survived. The remainder were unaffected.

Experiment 2. A group of 14 guinea-pigs of 250 g received a single injection of 1 c c of anatoxin of strain P W 8, precipitated with potassium alum. Schick tests 30 days later gave negative results. Thirty-three days after immunisation, 5 animals were injected with *gravis* toxin, one receiving 30, a second 40 and three others 60, 80 and 100 M L D respectively. Simultaneously five animals were given subcutaneously 1000, 2000, 4000, 6000 and 9000 million organisms of a living 24-hour culture of *gravis* strain 117. The large doses of toxin had no effect, but those animals which received 2000-6000 million bacilli showed infiltration at the site of injection and the animal receiving 9000 million died after 12 days. The post-mortem examination showed normal suprarenals and pleural effusion. The control animal, which received 10 M L D. of toxin, died with typical post-mortem appearances, as did the control which received 200 million organisms of living culture and died 22 hours after injection. Two days later 3 of the remaining immunised guinea-pigs were given 500, 700 and 1000 M L D respectively of *gravis* toxin, while the fourth received 20,000 million organisms of a 24-hour culture of *gravis* strain 117. The controls died and showed characteristic post-mortem findings. The animals which had received toxin reacted with a massive and cedematous infiltration at the site of injection but survived. The animal injected with living culture also had a marked infiltration and died 11 days later. Post-mortem findings were atypical. Diphtheria bacilli were not grown from the organs.

Experiment 3. A group of 18 guinea-pigs of 250 g was split into two subgroups of 9, of which one received one injection of 1 c c of alum-precipitated anatoxin from the strain P W 8 and the other three injections of 1 c c of unprecipitated P W 8 anatoxin at intervals of 8 days. Thirty-four days after the last injection, as a negative Schick test had developed, 3 animals in each subgroup received 300 M L D of *gravis* toxin. Three others in each subgroup received 3000 million bacteria of a 24-hour *gravis* culture and the remaining 3 the same amount of a 24-hour *mitis* culture. The animals in both subgroups behaved in the same way to infection and intoxication. In other words, the two methods of immunisation had proved of equal value. In both subgroups, however, the same observation was made, that animals receiving *gravis* toxin or

gravis type in guinea-pigs Antitoxic *gravis* serum and P W 8 serum give similar protection against infection and intoxication, provided they are administered in time It is difficult to judge whether the results of these investigations can be applied to the conditions governing diphtheria infections in children The experiments show, however, that in work on guinea-pigs the preparations in common use to-day for obtaining active and passive immunity against diphtheria are of value in controlling both infections with *gravis* strains and *gravis* intoxications

SUMMARY

1 The Gundel-Tietz medium was used for macroscopic diphtheria diagnosis with good results

2 Investigations during an epidemic in Kharkoff, predominantly *gravis* in type, yielded more than twice as many positive results by the macroscopic method as were obtained by the older Loeffler method This was presumably due to the frequent defect of granule formation in *gravis* cultures

3 In clinical cases the *gravis* type caused 72 per cent of the infections, the *mitis* 26 per cent, the *intermedius* 0.8 per cent and atypical strains 1.2 per cent

4 *Gravis* strains are more frequently present in both nose and throat than *mitis*

5 The three types proved stable under widely varied conditions of subculture

6. The *gravis* type was responsible for the vast majority of the clinically severe cases and was recovered during life from 96 per cent of all fatal cases

7 The presence of the *gravis* type of diphtheria bacillus was recognised *post mortem* in the organs of three cases investigated

8 Of the *mitis* strains isolated and tested in animals 38.6 per cent were avirulent, all *gravis* strains were virulent

9 Virulent *gravis* strains caused more rapid death of the guinea-pigs than did virulent *mitis* strains

10 Invasion of the internal organs took place in 40 per cent of guinea-pigs inoculated with *gravis* strains and only in 12.5 per cent of those inoculated with *mitis*

11 We were unsuccessful in making any observations which justified the conclusion that in the guinea-pig diphtheria bacilli are excreted by the tonsils

12 Precipitated and unprecipitated anatoxin immunises guinea-pigs against the toxin of *gravis* strains and against infection with *gravis* cultures

13 Large doses of living *gravis* cultures can overcome the active antitoxic immunity which has been developed in guinea-pigs

PREUNER, R	1936a	<i>Zbl Bakt</i> , Abt I, Orig, cxxxvi 463
"	1936b	<i>Ibid</i> , Abt I, Orig, cxxxvii 112
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ROBINSON, D T	1934	this <i>Journal</i> , xxxiv 551
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SHAPIRO, S L, AND RUK- VITCH, M V	1937	<i>Brit Med J.</i> , ii 373
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ZDRODOWSKI AND HALAPINE, C	1936	<i>Rev d'Immunol</i> , ii 221

+ agglutination just visible to the naked eye and \pm agglutination detectable only with a lens A saline control was included for each suspension

RESULTS

There was considerable individual variation in the response of the rabbits to immunisation but there was no great difference in the specificity of the sera of the rabbits immunised with 3-hour or 18-hour cultures The most satisfactory were those produced in animals inoculated with 18-hour cultures These could be absorbed with heterologous strains leaving type-specific agglutinin intact On the other hand absorption with heterologous strains of the sera prepared by immunisation with young cultures frequently resulted in the loss of homologous and heterologous agglutinin to an equal degree

Suspensions of overnight cultures killed by boiling gave satisfactory results in the agglutination test but slightly more specific results were obtained with killed suspensions of young cultures and these were used in most of the experiments

Sera prepared against three strains, S 11, S 80 and S 33, which had been found to show certain differences in precipitation tests (Cowan, 1938), were used in the early experiments About two-thirds of the strains of *S pyogenes* examined reacted in one of three ways Some agglutinated with S 11 antiserum only (type I), others with S 80 antiserum only (type II), while the remainder reacted with both S 11 and S 33 antisera (type III) Reciprocal absorptions were made and the results with the type strains are shown in table I

TABLE I Slide agglutination reactions of type strains and atypical strains of *S pyogenes*

Serum (type)	Absorbed with		Degree of agglutination with						
	strain	type	type strains			atypical strains			
			S 11	S 80	S 33	S 330	S 285	S 306	S 308
I	S 11	I	++++	—	++++	++++	+++	+++	++++
	S 80	II	—	—	—	—	—	—	—
	S 33	III	++++	—	++	++++	+	—	—
II	S 11	I	—	++++	—	—	+++	++	+++
	S 80	II	—	++++	—	—	+++	±	—
	S 33	III	—	++++	—	—	++	+	+++
III	S 11	I	—	—	++++	++++	+++	++++	++++
	S 80	II	—	—	++++	—	—	+++	++
	S 33	III	—	—	—	+++	—	++	++++

± to ++++ = increasing degrees of agglutination, — = no reaction

In a previous paper it was shown that by the precipitation reaction the majority of pathogenic strains fell into one group (A) while a few were placed in an ill-defined group (C). In the series reported here the recently isolated strains were not examined by the precipitation method but the stock strains of group C fell into

TABLE II.

Distribution of 105 strains of staphylococci among the serological types of S pyogenes

	Serological type				
	I	II	III	Atypical	Unclassified
<i>S. pyogenes</i>	28	16	15	26	1
<i>S. epidermidis</i>	0	0	0	0	15
Scurf staphylococci	0	0	0	0	4

type II, except one which belonged to type I. Since type II also contained group A strains it seems clear that the antigens responsible for the reactions in the two tests are not identical. The precipitation reaction suffers from the defect that unless the optimal proportions method is used the presence of multiple antigens in the crude bacterial extracts renders the results difficult to interpret.

DISCUSSION

Using agglutinin absorption methods Hine (1922) and Blair and Hallman (1936) divided pathogenic staphylococci into 3 types while Yonemura (1936) classified 324 strains into 9 serological types. It is likely that two of the types of the last worker were composed of saprophytic strains since they failed to produce hæmolysin, of his 311 hæmolysin-producing strains all except 9 fell into three types, in one of which a sub-type of 5 strains was recognised.

By slide agglutination it is easier to differentiate pathogenic from saprophytic staphylococci than by the usual method in agglutination tubes. About two-thirds of the *pyogenes* strains can be divided into three types while the remainder present difficulty on account of their atypical reactions with absorbed sera. The cross reactions may well be due to a sharing of antigens in varying proportions among the different types so that only quantitative absorptions would enable a true differentiation to be made. An analogous antigenic structure occurs in the *Brucella* group (Wilson and Miles, 1932), *Neisseria meningitidis* (Fildes and Baker, 1918), *Bact. flexneri* (Andrewes and Inman, 1919) and the *Salmonellas*.

Serological classification has a direct application in the study of epidemics of staphylococcal infections in hospital wards and in

air passages This argument no longer holds and other proofs will have to be sought if one is to attribute a pathogenic role to the *Hæmophilus* of the respiratory type The great importance of this question for the pathology of the respiratory tract becomes clear from the mere fact that strains of the respiratory type can be demonstrated, often in pure culture, in the great majority of sputa from patients with acute or chronic purulent bronchitis, acute capillary bronchitis and bronchopneumonia independent of epidemics of influenza From this one may conclude that this bacterial group is possibly the causative agent of common catarrhal purulent conditions of the bronchial mucous membrane (Mulder, 1938)

Much experimental and clinical bacteriological work, however, will be required to prove this working hypothesis and any contribution which shows the probability of the pathogenic role of the respiratory type in a given pathological condition will therefore be valuable

As the cultures of strains of the respiratory type are very similar to those of the R forms dissociated *in vitro* from type-specific S strains, some bacteriologists (Fothergill and Chandler, 1936) consider all sputum strains as R variants and do not attribute pathogenic properties to them Against this view, which is quite unproved, we think the following facts can be advanced (1) *Hæmophilus* of the respiratory type is found far more frequently in purulent sputum than are type-specific strains, and it would be unique in bacteriology for R variants to be found exclusively at the site of disease in so great a proportion of cases (2) The patients in whose sputum we found type-specific strains did not present an exceptionally severe bronchitic or bronchopneumonic process (3) As the most frequent and probably also the most parasitic sub-type of the type-specific strains is sub-type b, we should expect that in a large series of strains of the respiratory type, there would, if these were R variants, be many which would have the serological reactions of the R variants of sub-type b In our experience this is not the case, as is shown by the following investigations

*Capsular (type-specific) and somatic antigenic structure
of S and R strains*

An antiserum prepared in a rabbit against a type-specific S strain agglutinates the homologous S strain into coarse floccules on a slide at room temperature The dilution of serum up to which this slide agglutination is still possible varies, but with potent sera it may be as high as 1:20 Up to the present we have found this reaction to be type-specific In view, however, of experience with slide agglutination tests with various types of pneumococci

agglutinable only with difficulty, their titres remaining lower than those of the homologous R strains. Spontaneous agglutination of the controls, in our experience, is rare even with R forms. As diluting fluid we use 0.2 per cent saline.

In the lower dilutions of R serum the floccules are always small, whereas in the lower dilutions of S sera the type-specific discs are formed as described above with S strains of the same type.

We had certain difficulties in these agglutination tests. The final dilution obtained with a given serum and the homologous strain is not always the same on repeating the test (*e.g.* tables V and VI). Some strains give only a fine granular agglutination which is difficult to read. We do not know whether this should be understood as a pseudo-agglutination. It is a rare occurrence. On one occasion a freshly isolated R strain of sub-type b produced a fine granular agglutination, and 2½ months later it failed to do so any more. An old R strain of this sub-type likewise did not agglutinate (table VI).

*Cross agglutination reactions of the S and R forms
of various strains of sub-type b*

Table II gives the results of cross agglutination tests carried out with S and R sera and various S and R strains of sub-type b. They show that the somatic antigenic structure of the various strains is almost identical, that the R forms are agglutinated by the S sera to titre, and that the S strains are strongly agglutinated by the R sera. Strains b₂S and b₅S were less readily agglutinated.

TABLE II

*Cross agglutination of the S and R forms of various strains of
Haemophilus sub-type b by S and R sera*

Antiserum	Agglutination titre with various strains							
	b ₁ S	b ₁ R	b ₂ S	b ₂ R	b ₃ S	b ₄ S	b ₄ R	b ₅ R
b ₁ S	1600	1600	800	800				
b ₁ R (I)	1600	1600	800	800				
b ₂ S			800	800				
b ₂ R	12800	6400	400	3200				
b ₃ S	640	1280			1280			
b ₄ R	1600	3200				6400	12800	320 3200

S = smooth strain or corresponding antiserum

R = rough strain or corresponding antiserum

This and all other tests were done at 56° C

We have repeated these experiments with sera b₃S and b₁R and 5 R strains. Four of these were isolated from cerebrospinal fluids by Professor Kapsenberg and subcultured by him for years.

TABLE IV
Cross agglutination reactions of the S and R forms of type-specific strains of *Hæmophilus*

Anti-serum	Agglutination titre with various strains									
	aS	b ₃ S	b ₁ R	cR	eS	eR	fS	fR	gS	gR
aS	1280	160	0	0	1280	1280	0	0	640	640
b ₃ S	40	1280	1280	0	0	0	10	320	80	10
b ₁ R (II)	20	320	1280	640	0	0	320	160	80	320
eR	0	0	0	2560	0	0	0	0	0	80
eS	80	0	0	10	1600	3200	0	0	320	160
eR	0	0	0	160	6400	5120	0	0	640	640
fS	0	0	0	0	0	0	2560	2560	0	0
fR	0	0	0	0	0	20	1600	2560	0	10
gS	160	640	640	160	320	320	640	160	640	320
gR	0	5	320	160	0	0	0	0	320	640

*The diagnosis of R variants of type-specific strains of
Hæmophilus influenzae*

From the above observations we conclude that the diagnosis of the "R form of a type-specific *Hæmophilus* strain" is only justified when an antiserum prepared against the strain agglutinates strongly at 56° C both the R and S forms of the type-specific strain and the strain in question is strongly agglutinated by the anti-S and anti-R sera of the same type-specific strain

Elsewhere (Mulder, 1937) we gave instances of conditions in which it may be desirable to ascertain whether a *Hæmophilus* strain of the respiratory type is the R form of a certain type-specific strain. A further interesting example is the following

In the sputum of a patient with pneumonia, caused by pneumococcus type I, a type-specific *Hæmophilus* strain of sub-type e was found, the identification was kindly confirmed by Miss Pittman. A few days later we failed to find this type again but from the sputum was grown a non-type-specific strain of the respiratory type. The question now arose whether this respiratory type could be the R form of sub-type e. In the first place it was found that the respiratory type formed indole, whereas eS and eR did not, but this biochemical difference, in our opinion, would not be decisive

became more and more frequent, and he died on the sixth day after admission

A second bacteriological examination of the CSF cultured on aseptic fluid agar produced numerous small colonies of small Gram-negative coccobacteria, which, when received by us from the Neurological Department, did not grow on subculturing. Autopsy (Dr P. Stibbe) revealed acute purulent cerebrospinal leptomeningitis. In the lungs no inflammation was found. The nasal sinuses and middle ears contained no pus. The meninges on the convexity were translucent and seemed somewhat thickened. In the left Sylvian fossa there was a round yellow patch about 1 cm across, consisting of pus. At the base a flake of yellow pus was found on the chiasma, and in connection with this, also to the left and right on the temporal poles. The liquor was slightly turbid. In the lumbar portion of the spinal cord there were a few patches of yellow pus.

From the pus at the base of the brain we obtained a culture of a haemoglobinophilic bacterium contaminated with *Bact coli*. Gram-positive cocci were absent.

The colonies of the initial culture did not show iridescence by reflected sunlight or artificial light. Indole was not formed. Growth occurred only in the presence of X and V factors. The pathogenicity for rabbits by intravenous inoculation was not examined. Culture washings did not show precipitation with the type-specific serum against sub-type b nor was there type-specific slide agglutination at room temperature.

The question arose as to whether the organism was an R variant of sub-type b which had developed since isolation. Theoretically it is quite conceivable that this should happen, as in some S strains of sub-type b we have seen the S-R dissociation occurring after two subcultures. This supposition was less probable, because the strain Lorenzo did not form indole, while we know that the great majority of strains of sub-type b do so. One strain, however, which we have examined did not form indole, and Miss Pittman also informed us that she had found an occasional strain which did not.

Evidence that the organism was not such a variant was provided by cross agglutination tests with antiserum against the strain Lorenzo and with the anti-S and anti-R sera of strains of sub-type b (table VI).

The results indicated have been obtained repeatedly, and are sufficiently distinct to justify the conclusion that the strain Lorenzo is no R variant derived from a strain of sub-type b.

Absorption tests showed that the serum b_1R , exhausted by its own strain, retained its activity against the strain Lorenzo, and conversely that, when exhausted by the strain Lorenzo, it retained its power to agglutinate its own strain. This is further evidence that the strains b_1 and Lorenzo must be considered as quite distinct.

We have further tested the 5 strains R_1 , R_4 , R_5 , R_7 and

strain Lorenzo is probably not an R variant of any of the sub-types a, c, e, f or g. We cannot say this with the same certainty as we can with regard to the sub-type b, as we are not sure whether the somatic antigenic structure of the other sub-types shows the same homogeneity as seems to be the case with sub-type b. But we know that, of the remaining sub-types, only e has been isolated, and that once as a definite pathogenic organism—by Platt in a case of purulent meningitis. There is therefore almost conclusive evidence that the strain Lorenzo is of respiratory type.

TABLE VIII

Agglutination of the strain Lorenzo with the antisera of the type-specific strains a-g

Strain	Agglutination titre of different antisera									
	aS	b ₁ S	b ₁ R(II)	cR	eS	eR	fS	fR	gS	gR
Lorenzo	20	80	320	10		40		320		40

We would remark that it is necessary that other differences between R variants and *Hæmophilus* strains of the respiratory type should be sought. It may be possible that a further distinction will be furnished by a study of animal pathogenicity.

Summary

1 A case of purulent meningitis is described from the cerebrospinal fluid of which an indole-negative hæmoglobinophilic organism of the respiratory type was grown in the first culture.

2 Evidence is adduced to show that this strain cannot be considered as a rough variant of a strain of Pittman's sub-type b.

3 The diagnosis of rough variant may only be made when the strain is strongly agglutinated at 56° C by an anti-S and anti-R serum of a type b strain, and the antiserum to the strain in question also agglutinates strongly the S and R forms of sub-type b.

4 This observation makes it quite probable that in rare cases the *Hæmophilus* organisms of the respiratory type can cause purulent meningitis. This again is of some importance for the consideration of the pathogenic significance of this group in the respiratory passages, where it causes, in the author's view, common purulent bronchitis.

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| KAPSENBERG, G | 1929 | <i>Ned Tydschr Hyg</i> , iv. 153 |

found in the perivascular connective tissue and about capillaries in the parenchyma at all ages

Adipose tissue cells in varying numbers were present in the interstitial tissue at all ages and in almost all cases. They were scanty in paraffin preparations in 2 of 10 subjects aged up to 1 day, in 1 of 11 subjects between 1 day and 3 months, in 5 of 9 subjects between 3 months and 1 year, in about half between 1 and 5 years, in all but 2 between 5 and 10 years and in all but 1 between 10 and 20 years. In a few of these young subjects the fat cells were relatively numerous but according to the estimations of parenchyma made for the statistical paper (Gilmour and Martin) in none was the volume of parenchyma reduced below 69 per cent whereas in adults a much greater reduction resulted from adiposity. In osmic acid preparations or in frozen sections stained with Scharlach R fat cells were seen in all subjects of 4 months and over, but in none of younger age (table, p 203). In general the interstitial fat increases from infancy onwards and severe degrees of adiposity are not seen before the age of 30. Of 69 subjects in whom the estimated parenchyma occupied 60 per cent or less of the gland and the interstitial tissue was predominantly fatty, 29 were recorded as being fat, stout or obese, 27 as moderately or well nourished and 13 as wasted, but in the latter the fat did not reach the higher amounts. It was shown in the statistical work (Gilmour and Martin, table X) that at least in age groups 11 to 20 and over the fat in the interstitial tissue is greater in females than in males. Adiposity begins in the perivascular connective tissue but fat cells singly or in groups may also lie in the parenchyma, arising from small spindle cells upon the reticulum around the capillaries. Adiposity is evenly distributed or is greater in parts, especially at the ends of the glands. It is important to recognise the continuity of the capsule over extremely adipose ends of glands since small islands of parenchyma left in it may otherwise be regarded as accessory tissue. Rarely, great adiposity causes pressure atrophy of parts of the parenchyma, the parenchymatous trabeculae being few and composed of poorly differentiated cells, such parts resemble closely very adipose involuted thymic tissue. Wasting of the fat is occasionally seen. The adipose tissue cells then sometimes remain large and have an abundant vacuolated eosinophil cytoplasm, as in the so-called "fat glands" of wasted children. foci of such cells in adults may suggest oxyphil cell groups. Usually, however, the cells as they lose their fat diminish in size to end as spindle cells. Wasting of fat is frequently seen in inflammations of the parathyroids.

In places where the perivascular connective tissue is dense and hyaline it usually includes spindle cells of varying size, often reaching $60 \times 6 \mu$ and containing much finely particulate yellow-

In 23 of 428 subjects (5 per cent) paraganglion tissue was found on the surface, in the hilum or deeper in one or two of the glands (fig 2). The tissue was arranged in small rounded masses in which stellate basophil cells lay in a whorl of spindle cells and capillaries. In morphology and arrangement the cells were identical with those of the carotid body. The number of such paraganglia in any gland was variable. In 2 subjects the position of the glands containing them was not recorded, otherwise they were in upper glands only, occupying both in 3 subjects. Mention was made in the embryological paper (Gilmour) of paraganglion tissue in the hilum of an upper gland of an infant two days old and in the hilum of the thyroid gland of a foetus of 120 mm.

Fischer (1911) described the elastic in the capsule and stroma, Petersen (1903) saw it abundantly in the capsule but could not see it in the veins, von Verébely (1907) described it as limited to the large arteries.

Yanase (1908) described valves in the veins especially in the hilum and gave an illustration. Oppenheimer (1911) also described them but in his illustrations they look like artefacts rather than true valves. I could recognise many valve-like artefacts due to tearing of the endothelium or branching and bending of veins.

Sandström (1879-80) could not find lymphatics but Petersen (1903) and Oppenheimer (1911) described them in the perivascular tissue.

The blood supply has been described by various authors. Sandström stated that the inferior thyroid artery supplied the glands and Welsh (1897-98) confirmed this, stating that if the glands are aberrant they receive supply from the nearest vessels. Halsted and Evans (1907) showed that the glands are supplied by a small single special artery arising from the muscular, glandular or oesophageal branches of the inferior thyroid artery. The upper gland might receive the vessel from an anastomosing ramus connecting the inferior and superior thyroid arteries. Curtis (1930) showed that all the parathyroids can be injected from the aorta after ligation of the inferior and superior thyroid arteries, thus demonstrating a good collateral circulation and he said that these four vessels can be ligated in operations without damage to the glands.

Andersson (1894) described in two animals a perivascular plexus of nerves from which fibres ended in a point or with a swollen end among the epithelial cells. According to Braeucker (1922-23) fine nerve fibres from the vagus and sympathetic run into the glands with or apart from vessels. Petersen could not find medullated fibres. Rhinehart (1912) stated that the nerves are all non-medullated, that they enter as a plexus round the vessels and are derived from the same source as the thyroid nerves. Herrman (1936) saw macroscopically a small branch of the recurrent laryngeal nerve enter each gland. de Winwarter (1930) described nerve endings like Pacinian corpuscles in the parathyroids of guinea-pigs.

Mast cells were described in the parathyroids by Erdheim (1903) and by most subsequent authors.

Sandström described fat in the interstitial tissue and Benjamins (1902) saw it already in the foetus. Erdheim (1903), however, did not see it till the 5th year while Gossmann (1927) recognised it first at 6 weeks. Gossmann (1927) and Fischer (1911) saw very adipose glands in cachectic patients.

Erdheim (1904b) described the presence of iron pigment in the perivascular connective tissue of infants. He also (1907) first described yellow-

nucleus to involve almost the whole cell, a very little granular protoplasm may remain under the cell membrane. The cell membrane is invisible or inconspicuous in the preceding types but prominent in water-clear cells. The cells vary in diameter from 7 to 15 μ , average about 10 μ . The dropsical appearance of transitional and water-clear cells in tissue fixed in watery fixatives is due to the removal in solution of paraplasmic glycogen.

Types of principal cells at different ages In the embryo and in early foetal life all the principal cells are water-clear. Some transitional cells were seen in the oldest foetus (190 mm.) examined (Gilmour). At and after birth water-clear cells are always present but rarely predominate. They may be found in ill-defined areas or in well defined nodules, even in glands that otherwise contain few such cells. At birth and up to 1 year transitional cells predominate as a rule, but water-clear cells are numerous and predominate more often than in later years. Thus in the series of 357 subjects in whom there was no renal disease nor gross anatomical or histological variations from the normal in the parathyroid glands, 29 were under 1 year, and in 5 or 17 per cent water-clear cells predominated. In the age group 1-19 the relative number of dark and early transitional forms increased, and in none of the 47 subjects did water-clear cells predominate. In only 5 or 1.8 per cent of 281 subjects of from 20 to 80 years of age did these cells predominate. Of the 10 subjects in whom they predominated after birth the youngest was aged 12 hours and the oldest 67 years, most were well nourished but 3 were wasted. The predominance was sometimes present in only one or two of the four glands, and the remaining glands occasionally contained dark principal cells alone.

Rosarot principal cells Hæmatoxylin has little affinity for the cytoplasm of principal cells compared with pyronin, methyl blue and methylene blue. In most normal glands, however, the cytoplasm can be stained with hæmatoxylin and eosin a blue-purple or even a grey-blue in clear contrast to the pure pink and red of pale and dark oxyphil cells, provided the method given in the section on staining (p. 195) is followed. In some glands, on the other hand, the cytoplasm of a variable number of principal cells, usually in groups, has a greater affinity for eosin, sometimes so great that the cytoplasm remains a distinct pink when the eosin has been washed completely out of the granules of some of the pale oxyphil cells. The pink in such eosinophil principal cells is, however, tinged slightly with blue when examined in daylight and is consequently not so pure, or as artists say "warm," as in oxyphil cells. To indicate principal cells with exceptional eosinophilia the term "rosarot," used erroneously by Getzowa (1907) for dark principal cells, may be adopted. The pink colouration is naturally more conspicuous in cells with denser cytoplasm. The nuclei of rosarot

brightly, in others smaller, more sparse and less deeply stained. The latter are probably early or transition forms. Very rarely the cytoplasm is beset with minute vesicles which can only be seen with a $\frac{1}{12}$ in objective, and granules cannot then be recognised between the vesicles. Larger vacuoles are frequently seen in paraffin sections, and these are occupied by lipoid in frozen sections (p. 202). In two cases partial dropsy of some of the cells was found resembling that in transitional principal cells. In one of these, water-clear cells predominated and it looked as if the dropsy had extended to some of the oxyphil cells. The cell membrane is well defined in pale oxyphil cells but is not so thick and prominent as in water-clear principal cells. Stained with Mallory's acid fuchsin and aniline blue or Masson's trichrome the membrane is blue in contrast to the red granules, while with pyronin methyl-green it is pink in contrast to greenish blue granules.

Dark oxyphil cells most often lie within groups of pale oxyphil cells but are also found scattered among the principal cells (fig. 1). Within these groups they tend to be distorted by pressure, developing concave surfaces and a more or less crescentic shape. Such cells measure often $15 \times 5 \mu$. Amongst principal cells they are polygonal or rounded, and measure $7-14 \mu$ in diameter. The nuclei resemble those of the pale oxyphils, usually of the smaller sizes, and are more often pyknotic. Oxyphil granules similar to those in the pale oxyphils are seen in some of the cells but the intervening cytoplasm is homogeneous to a variable degree and in most cells completely obscures the granules. Dark oxyphil cells are obviously formed by a change in the cytoplasm of the pale variety. The transition is best followed in sections stained with Heidenhain's iron hæmatoxylin. The ground substance of the cytoplasm becomes progressively darker and in the end homogeneous and black. The granules soon become invisible. Finally no structure is seen except a small vacuole or pigment sphere. A similar change is seen with Mallory's phosphotungstic-acid hæmatoxylin which stains the oxyphil granules a distinctive blue. The cytoplasmic ground substance from being uncoloured becomes progressively bluer until it is dark blue and homogeneous. The granules are perceptible for a longer time than with Heidenhain's hæmatoxylin but ultimately are obscured. With Ehrlich's hæmatoxylin and eosin a deep eosinophil change appears, sometimes affecting first only part of the cytoplasm but soon becoming diffuse and leading to early disappearance of the granules. The final result with all stains is a homogeneous, hyaline, refractile oxyphil mass with a pyknotic nucleus. Fragmentation of both nucleus and cytoplasm can then occur. Fragmented cytoplasm without a nucleus can not infrequently be seen. With Weigert-Gram the dark oxyphil cells tend to retain the stain and may be frankly Gram-positive.

his painting reproduced in fig 1 (for which I am indebted to Professor Turnbull) the late Mr Ford unfortunately used artificial light with daylight the cytoplasm of the deep oxyphils in this section is purple-red, that of the principal cells almost blue. If the glands are not perfectly normal and rosarot principal cells are present these can usually be differentiated in daylight because of their being tinged more or less with blue so as to be a colder pink than the oxyphil cells. This distinction in colour is slight, however, so that the possible presence of deeply eosinophil rosarot cells prevents the stain from being a perfect method of differentiation, it is still less reliable in abnormal parathyroids such as the hyperplastic glands in generalised osteitis fibrosa.

Turnbull (Hunter and Turnbull, 1931-32), for precise differentiation of the cells in formal-fixed material, used a solution of Unna-Pappenheim's methyl-green pyronin according to the formula of Schmorl (1928). He had found this solution of great value in determining the degrees of basophilia of the cytoplasm of the cells of the marrow. The stain was applied for half an hour or longer, blotted, dehydrated with absolute alcohol as rapidly as possible, cleared with xylol and mounted in balsam. The granules of the pale oxyphil cells were stained pale greenish blue and their cell membranes pink. Occasionally more deeply red particles of cytoplasm were attached to the cell membrane or lay free within the cell body. The dark oxyphils were stained dark blue-green and a pink cell membrane was very rarely shown. The cell membranes of the principal cells were stained pink and the particles of cytoplasm pink or red, the dark principal cells being deep red. After a solution made up in 1922 had been used up we failed to obtain satisfactory results with similar solutions made with pyronin and methyl-green from various sources. Recently, however, Mr John King has obtained excellent results by staining for 15 minutes with the Unna-Pappenheim mixture according to the formula of McClung (1929), differentiating for five seconds or longer with carbol-water as used in the formula, blotting dry with filter paper and mounting immediately in Gurr's medium. The sections may be stained for a longer time and then longer washing in carbol-water is required to remove the excess of green. Differentiation is controlled with the microscope. The contrast between the green or greenish blue of the oxyphil cells and the pink or red of the principal cells is very sharp. Principal cells which appear rosarot with hæmatoxylin and eosin are stained pink or red. We have found the method to differentiate with certainty and constancy oxyphil and principal cells.

In Mallory's phosphotungstic-acid hæmatoxylin the oxyphil granules are sharply defined and usually but not invariably stained dark blue.

The long methyl blue and eosin method of Mann (1902) preceded by Lugol's solution sometimes differentiates the cells very clearly in formaldehyde material, but we have not obtained constant results. The preliminary treatment with Lugol's solution for 15 minutes, decolourisation in 95 per cent alcohol for at least 1 hour, and washing in tap-water are taken from Dr Dorothy Russell who has used this method with excellent results for staining the pituitary gland by Mann's long method.

Mallory's acid fuchsin and aniline blue according to the method of Crooke and Russell (1935) has occasionally given good results, the oxyphil granules being golden, but has proved even more uncertain than Mann's method.

Welsh (1898) gave the first good description of the cells of the parathyroids and applied the name "oxyphile" to the eosinophil granular cells which certainly had been seen before by Sandstrom (1879-80). The remaining cells Welsh called principal cells and he recognised several varieties

principal cells, but mention has been made of appearances in routine paraffin sections suggesting its occasional presence in oxyphil cells. Further, oxyphil cells are difficult to recognise in sections stained by Best's method unless they are in masses and it is impossible to deny that some oxyphil cells may contain a little glycogen. It is not as a rule evenly distributed. Its amount and position correspond to dropsical areas in the cytoplasm. Thus the greatest amount was found in water-clear cells and the next in transitional cells. It was seen in the cells of columnar-celled alveoli (p 208) and cystic vesicles (p 211) in some cases and was frequent within the lumen of the former.

Petersen first described glycogen in the parathyroids and nodules of cells rich in glycogen. According to Yanase, Guizzetti had described its absence from oxyphils and its abundance in children, and this Yanase confirmed. Kurokawa (1925) and Turnbull (Hunter and Turnbull, 1931-32) also found it absent in oxyphils in normal glands but the latter found it in pale oxyphil cells in an enlarged parathyroid in generalised osteitis fibrosa.

Pigment

In paraffin sections accumulations of pigment are seen in many cells of all glands of adults. As a rule only one is present in a cell but occasionally there are two and rarely three. Their diameter varies from 0.5 to 4 μ but is usually about 2-3 μ . They are usually round, but when small are often of irregular shape. The number varies considerably in different subjects and in parts of the glands of the same subject. Their density is also variable. Most commonly they are lemon yellow, pale and vacuolated, but with increasing density they become darker until finally they form solid yellow-brown spheres. Vacuoles are frequently seen in them, which vary in size and number and may be present even in examples only 1 μ in diameter. The pigment may be present only as a ring or crescent around a large central or eccentric vacuole, or it may form a capsule around and a net between a complex of vacuoles. The number of vacuoles decreases with increasing density of the sphere. These accumulations of pigment are most conspicuous in oxyphil cells and in these the darkest and most solid are seen. In water-clear cells they may appear suspended in the clear cytoplasm. In oxyphils and dark principal cells there may be a narrow clear halo around some of them, rarely a condensation of the cytoplasm like a capsule surrounds this halo, suggesting a bird's eye with the yellow sphere for pupil.

This pigment was seen in all subjects of 4 years and over in whom it was looked for, all available material between the ages of 4 and 20 being examined closely. Frequently under the age of 4 years very minute yellow granules were seen but they were too small to identify with certainty as pigment. In children the groups

show one or more clear rounded spaces evidently occupied by a clear fluid. Ring or crescent forms are caused by a large central or eccentric vacuole. Even the ring or crescent may have many smaller vacuoles. Sometimes a complex of vacuoles up to $7.5\ \mu$ in diameter is seen in which the lipid forms only a capsule and inter-vacuolar net. Morphologically the lipid is identical with the pigment except that the size of the lipid globules is often slightly larger.

Relation to yellow pigment In order to ascertain the relationship between the lipid and the spheres of yellow pigment drawings were made of suitable groups of lipid globules in osmic-acid-fixed preparations, the size and position of any vacuoles in them were indicated and the individual globules measured with an ocular micrometer. After removal of the blackened lipid by turpentine the sites occupied by the globules were examined again and pigment spheres were then usually revealed in them. The pigment spheres were seen to have been blackened and usually had the same shape as the lipid and identical vacuoles, but many had also been surrounded by lipid, since their diameter was often $1-2\ \mu$ less than that of the corresponding lipid globules. Such reduction was accompanied by the appearance around the pigment sphere of a clear halo which had not been seen around the blackened lipid globule. The removal of the lipid sometimes revealed additional vacuoles. These had probably been present in both the lipid and the pigment sphere but had been obscured by the osmic acid blackening. Some of the lipid, especially the small globules and granules, was dissolved away without revealing any underlying pigment.

It is seen therefore that the lipid is closely associated with the pigment but may also surround it. Whether the pigment is in a fluid state in the lipid could not be determined, but probably it is solid, since a fluid would diffuse and always occupy the whole lipid globule. It is possible that lipid-soluble lipochrome held in solution changes after some time into an insoluble lipoidaffin pigment. A similar origin was suggested for the pigment in the spindle cells in perivascular fibrosis (p. 189). No pigment spheres were unblackened in osmic preparations, but in paraffin sections of formol-fixed tissue the pigment was not blackened by treatment with osmic acid. It can be presumed, therefore, that all the pigment, even when forming dense solid brown spheres, was associated with some lipid.

Chemical nature The lipid was isotropic and gave a negative Schultz reaction for cholesterol. In only one subject, with abnormal glands, was cholesterol seen, it was in phagocytes in small cysts, it gave a positive Schultz reaction, formed anisotropic liquid crystals and was coloured pink or purple with Nile blue sulphate.

Apart from the frequent staining of pigment in paraffin section by Scharlach R, the oxyphil cells usually took up a slight diffusely yellowish colour. This was also seen in frozen sections.

The chief differential features of the lipoid substance can be summarised as follows. It was soluble in acetone in the cold, isotropic and free from demonstrable cholesterol. It was stained by Scharlach R, blackened by osmic acid, stained deep blue by Nile blue sulphate and stained black by the methods of Smith, Dietrich and Weigert-Pal. It required much longer time to saturate than fatty substances demonstrated by Ciaccio's method, but less than the great bulk of the neutral fat or mixed neutral fat and fatty acid in the adipose tissues. According therefore to the present, undoubtedly inadequate, knowledge of the histochemical properties of different fatty substances it would appear to consist chiefly or entirely of free fatty acids.

Amount at different ages and distribution. As shown in the table lipoid in minute granules was first seen at 6 months, and next at 5 years. Hollow centres were first seen at 8 years. The absence at earlier ages is, however, open to criticism since in most cases frozen sections stained with Scharlach R were used and this method is unsatisfactory for revealing small amounts and small granules. It was thought that the presence of fatty degeneration in other organs in 3 cases might have had some causal relationship with the rather numerous granules and small globules of lipoid present. In the subject, aged 6 months, in whom lipoid was first seen in the cells no microscopical preparations were made of other organs and the presence or absence of fatty degeneration in them could not be decided, but the child died, after an operation for hydrocephalus, in status epilepticus with a high temperature, and bronchopneumonia was found at necropsy, so that fatty degeneration may well have been present. It would require healthy subjects and osmic-acid-fixed preparations to decide the first appearance of lipoid. The average full amount of lipoid was first seen in a subject aged 25, but the most abundant lipoid in largest globules occurred in older adults.

Many glands of adults show small areas in which the lipoid is scanty, absent, or in the form of numerous minute granules. These are the proliferation foci of certain authors.

Lipoid was always less abundant in oxyphil cells than in principal cells.

In colloid and cystic vesicles (p. 210) and in columnar-celled alveoli (p. 208) the lipoid shows a distinct tendency to lie in the cytoplasm between the nucleus and the free border of the cell and, in the case of parenchymatous trabeculae two cells wide, on the side of the nucleus towards the centre of the trabeculae. In columnar-celled alveoli and colloid vesicles lined with cubical cells

Kurokawa (1925) first saw it at age 4 Arndt (1922-23) and Gossmann (1927) in man and Ohmori (1929) in guinea-pigs stated that it was stained by the Smith-Dietrich method, Arndt and Ohmori that it was Ciaccio-positive, in part at least according to Arndt Gossmann, Arndt and Ohmori frequently found anisotropic substances and stated that cholesterol may be present Erdheim (1903) could not find anisotropic lipid in the parathyroids

Damsch (1924) stated that principal and oxyphil cells of old people contain fat which is resistant to fat solvents This was probably the pigment spheres Damsch was apparently the only author to note the light staining with sudan of oxyphils in paraffin sections

IV GENERAL ARRANGEMENT OF THE PARENCHYMA

It is possible to distinguish four types of gross structure

1 *Compact* (fig 3) The gland or part of the gland consists of a solid mass of cells perforated here and there by relatively few



FIG 3 —Compact gross structure, parenchyma shaded

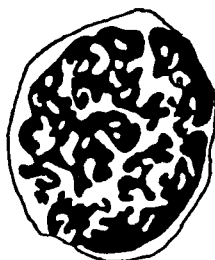


FIG 4 —Coarsely trabecular gross structure

and small canals containing vessels and scanty perivascular connective tissue

2 *Coarse trabecular* (fig 4) The parenchyma consists of solid branching and anastomosing trabeculae many cells thick This type only differs from the compact in that the perivascular connective tissue is more abundant Swelling of cells from glyco-genic distension (water-clear cells) or increase in number from hyperplasia tends to produce glands of compact type, while adiposity of the stroma produces trabecular glands

3 *Lobular* (fig 5) The parenchyma of glands or parts of glands is split into angular masses by scanty areolar tissue

4 *Large acinar* (fig 6) This type resembles the lobular but the masses are rounded It is not often seen and is usually confined to one side of a gland

The parenchyma forming these types of gross structure is

and anastomosing narrow trabeculae usually two cells wide. In children and infants the capillaries are fewer and the trabeculae wider, being 3-6 cells wide. In hæmatoxylin and eosin sections, if the reticulum is almost confined to the walls of the capillaries, the trabecular substructure may be indistinct but oedema and especially congestion may render it prominent. If there is much free reticulum extending from that around the capillaries oedema makes more obvious a fine trabecular and small acinar substructure. Most free reticulum is found in glands or parts of glands where a small acinar substructure predominates, the acini being surrounded by a layer of reticulum in which capillaries are found only at intervals (fig. 8)

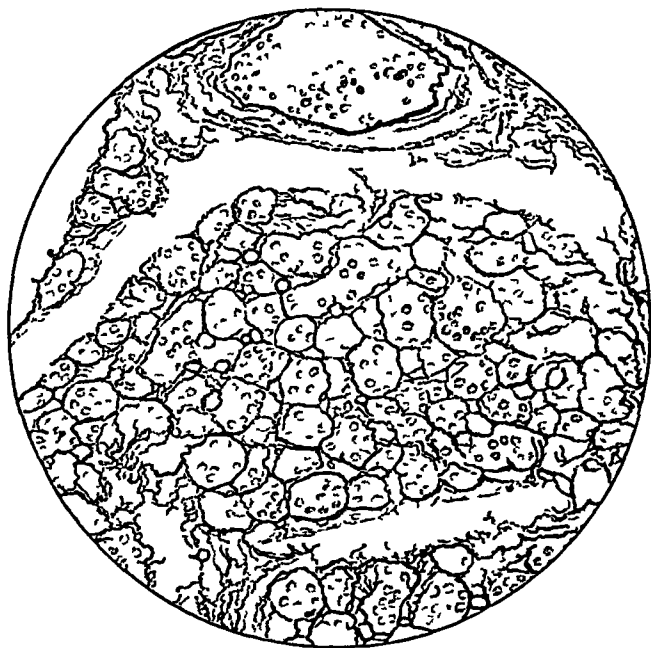


FIG. 8.—Drawing of reticulum ♂ 58 Laidlaw's impregnation $\times 240$

The small acini, usually two cells wide, often appear like independent structures but they are probably united. To produce considerable areas of small acini, however, trabeculae would have to be much altered in shape, if not segmented, by the development of abundant free reticulum. Elaborate reconstruction methods would be necessary to decide whether such acini are free or connected. Reticulum separating individual cells was seen only in a few patches in adult glands and was considered to be an expression of fibrosis. In oxyphil cell masses the reticulum is confined to walls of capillaries. Colloid vesicles are surrounded chiefly by free reticulum with capillaries only at intervals.

fixed in Gatenby's fluid the lipoid in the nodule was again very scanty and formed of small droplets. A femur examined in each of the six cases showed osteoarthritis in three and no bony abnormality in the others. Five subjects were female. All were between 44 and 80 years of age. It is probable that these nodules of dark and transitional principal cells have no relation to bone disease but have the same unknown significance as nodules in the thyroid, suprarenal and pituitary glands in adults.

Sandstrom (1879-80) described the compact and trabecular structure to which Kohn (1895) added the lobular type. Bergstrand (1919-20) described reticulum around the capillaries from which fibrils run off to surround the follicles with a complete lattice-work. He pointed out that there is no *membrana propria* such as Sandstrom had described. He could not find reticulum branching from the capillaries in the first years of life.

Erdheim (1907), Todyo (1912) and Danisch (1925) described, in normal glands of adults, foci characterised by scantiness of lipoid, the lipoid being in the form of small or minute, even if numerous, granules. They regarded the foci as areas of proliferation. The foci were best revealed by osmic acid fixation. Significance was attached to an abnormal number and size of such lipoid-poor foci in certain diseases of bone, even when the glands were of normal size. Bergstrand (1920-21) did not find them in unenlarged glands and considered that they may have been confused with oxyphil cell masses, which are always poor in lipoid, or with areas imperfectly penetrated by osmic acid. He considered that the only structures worthy of the name of proliferation foci were discrete nodules of principal cells such as Erdheim (1907) described in 3 adults.

V INCONSTANT STRUCTURES IN THE PARENCHYMA

Columnar-celled alveoli

A columnar-celled alveolus (fig 9) is a lumen lined with a layer of short or tall columnar cells and resembles an alveolus of a secreting gland. Usually in single sections they appear to be unconnected with the neighbouring gland but serial sections show them to be within trabeculae of solid parenchyma. The lumen is sharply outlined as a rule, its diameter varies from $3\ \mu$ to $40\ \mu$ but is usually about $10\ \mu$. The lining cells are essentially the same as in the rest of the gland except as regards size and shape. They are usually transitional, rarely water-clear, principal cells and occasionally an oxyphil cell lies among them, a complete lining of oxyphil cells is rare (figs 10 and 12).

The alveoli usually occur in single or multiple groups (fig 10) of about 3-15. Scattered single alveoli may be present, with or without groups elsewhere. The groups tend to lie near the surface of the glands, occasionally in the form of discrete nodules, or they lie in a nodule of water-clear cells. Within the groups or in small areas elsewhere are frequently found small acini (fig 10) and slender trabeculae composed of short columnar cells with basal



Pseudo-alveoli.

Another type of alveolus is frequently seen and although it is pathological it is mentioned here since it might be confused with a secretory alveolus. Pseudo-alveoli (fig 14) lie characteristically in the centre of one or perhaps two glands in a subject. They are somewhat larger than true columnar-celled alveoli and their outlines are not so sharp and regular. The lining cells are usually cubical or short columnar and arranged in a palisade. The content is usually unstained except for some granular or reticular coagulum, a few red corpuscles and perhaps a desquamated cell or two. Phagocytes containing iron were seen in or about them in two subjects. They are formed by mechanical separation of a column of cells by the escape of tissue fluids in cedema and congestion, which are frequently recognisable in the affected glands. Similar pseudo-alveoli may be formed by frank hæmorrhage. They are not so frequently seen as true columnar-celled alveoli and were found in only 33 of 428 subjects (8 per cent). However, the number in any affected area is greater than that of columnar-celled alveoli in their focal groups. An unusual expansion of the lumen occasionally seen in columnar-celled alveoli is probably due to such a pathological cedema fluid being added to the secretory fluid.

Trabeculæ of columnar cells

Slender trabeculæ two cells wide were described in the general arrangement of the parenchyma as making up the bulk of the substructure of glands. Occasionally, especially near the surface of glands, the cells become large and columnar while the nucleus is basal. This is most conspicuous in pathologically enlarged glands or in normal-sized glands where columnar-celled alveoli are numerous. Trabeculæ of this structure probably indicate increased secretory activity and precede the formation of alveoli.

Colloid vesicles

Two types of vesicles containing colloid-like material occur—the true colloid vesicle and the cystic vesicle.

True colloid vesicles are small, spherical or somewhat oval, are lined with a sharply defined regular row of cells and contain a deeply eosinophil homogeneous coagulum (fig 15). They arise, as has been mentioned above, from columnar-celled alveoli by a transformation of watery into colloid secretion and by shortening of the cells as the globule of colloid increases in size. They are probably also formed by the enlargement of colloid droplets formed between polygonal cells, when such a droplet reaches about 15-20 μ in diameter the formation assumes the appearance of a small

The cysts often lie isolated in, or project into, the interstitial tissue or capsule (fig 16). Usually, however, they lie completely within the parenchyma (fig 17). In groups in the latter position it is frequently possible to follow their development. Transitions are found between small deposits of a pale coagulum between polygonal cells to large, irregularly shaped cysts, the most irregular resembling a lake with long promontories projecting into it (fig 17) or a collection of elongated lakes separated by isthmuses. Such multilocular forms are found only in the superficial parenchyma.

The content is reticular, globular, finely or coarsely vacuolated or homogeneous, and seldom fills the vesicle completely. It is often fragmented. Usually it is stained very faintly or less deeply than the colloid in true colloid vesicles. Occasionally a faintly stained coagulum contains small, deeply eosinophil globules. Rarely, eosinophil fragmented contents are surrounded by a faintly haematoxyphil coagulum which gives the specific reactions of mucin. A homogeneous content as deeply eosinophil as that in true colloid vesicles is unusual. A few vesicles are apparently empty. In 100 subjects no crystalline structures were detected in the vesicles in paraffin sections examined with the polarising microscope.

The lining shows much greater variation than in true colloid vesicles. A single vesicle may be lined in part with greatly flattened cells and in part with cubical or spheroidal cells, while in another part there is no sharp boundary but the pale content infiltrates between the adjacent polygonal cells of a mass of parenchyma. The vesicles which lie isolated in the interstitial tissue or capsule are frequently bounded by a wall of hyaline fibrous tissue and the large racemose vesicles within the parenchyma often have a similar wall in places. Vesicles in groups are occasionally separated from one another by strands of hyaline fibrous tissue. Such fibrous walls are usually lined with a single layer of greatly flattened cells, the lining being often absent in places, but a lining of water-clear cells is frequent. Very rarely the lining includes one or two oxyphil cells.

Unilocular cystic vesicles are usually much larger than true colloid vesicles but seldom exceed $300\ \mu$ in diameter. Multilocular forms may occupy one or two mm.

Cystic vesicles are more common in the lower parathyroids. In 11 out of 428 subjects they were visible to the naked eye and all such lay in the lower glands. In a recent subject, however, typical examples were seen projecting from the surface of an upper gland. Within the glands there are three types of distribution all of which are common.

(1) Vesicles scattered throughout the gland, either all discrete or in places in groups.

(2) Vesicles deep in the gland. These are frequently lined with



stained in a few small, newly formed cystic vesicles. The apparatus was large, suggesting activity, but abnormal in structure. It is probable that most if not all cystic vesicles are the result of an abnormal and apparently functionless activity.

VI THE GOLGI APPARATUS

Some parathyroid glands were obtained from thyroids shortly after surgical removal and the Golgi apparatus was impregnated by Da Fano's silver method. Only a few glands were obtainable and the preparations were imperfect, showing impregnation only in small patches, while deposit was frequent. However, from examination of the impregnated areas some conclusions can be drawn.

In areas where the cells are polygonal and arranged in trabeculae the apparatus forms a net or tangle with one or more projecting processes and usually shows no definite localisation in the cell (fig 18). Sometimes when well defined trabeculae two cells wide are present the apparatus shows a predilection for, or the bulk of it lies in, the part of the cytoplasm furthest from the surface of the trabecula. In the few columnar-celled alveoli obtained the apparatus lies between the nucleus and the free border of the cell (figs 19 and 20). It is not obviously increased in bulk but appears to be more elongated than in polygonal cells. In colloid vesicles it is not enlarged, and occupies a similar position between the nucleus and the free border. A process may run round one or other side of the nucleus. In the few cystic vesicles seen the apparatus is atypical, especially in small and probably newly formed vesicles. It is irregular in shape and often enlarged. In some cells it forms a large solid mass of irregular outline, in others it consists of one or two processes which are irregular in their course and which sometimes extend from a mass and may form a loop (fig 21). The apparatus never forms a net as in polygonal cells of trabeculae. No definite Golgi apparatus was seen in oxyphil cells, even though these lay in areas where good impregnation was present in surrounding principal cells. The situation between nucleus and free border and the increased length of the apparatus in the cells of columnar-celled alveoli probably indicate secretory activity. The large size and atypical form in cystic vesicles probably indicates activity of an abnormal kind.

Courrier and Reiss (1922) found that in cats the apparatus lies in the side of the cell opposite that applied to the connective tissue septum. Cowdry (1922) said that the apparatus is reticular, shows great variability in localisation in the cytoplasm and is not definitely polarised for secretion.

The mitochondria were not investigated in this work. In the horse, Bobeau found the mitochondria arranged around the lumen when this was present.

lined with cubical ciliated epithelium attached to a lobule of thymus IV. From the parathyroid IV a process jutted out to this tube but did not fuse with it. Groschuff (1900) in a 4.4 cm. foetus saw a thymus IV close to a parathyroid IV, and a small tube-like cyst arose from the end of the thymus furthest from the parathyroid and passed into the thyroid. Cilia were apparently absent. Herrmann and Verduin (1899) described less precisely similar cystic spaces associated with upper glands in foetuses. These cysts or tubes were probably persisting portions of thymus IV when represented by a tubular diverticulum, the diverticulum having partially or completely failed to differentiate into thymic tissue. Erdheim (1904a) described a cyst which probably belonged to type 1. In a woman of 85 with left unilateral thyroaplasia a cyst was found beside the left upper parathyroid. It was lined with epithelium arranged in 1 or 2 layers and ciliated in places. Salivary glands of typical structure opened into it by ducts.

Type 2 Cysts visible to the naked eye, associated with the upper parathyroids and derived from thymus IV

Three cysts were seen beside upper glands in subjects aged 65, 49 and 41. They closely resembled each other. The largest measured $1.8 \times 0.6 \times 0.4$ cm. The parathyroid fitted like a cap upon two. They were multilocular, two to the naked eye, the third microscopically, but the loculi communicated freely. In two the content was milky and consisted microscopically of minute colloid-like balls in a pale staining reticular or granular coagulum. The fragmentation of the content probably caused the milky appearance. The epithelium in places was in a single layer but usually in several layers and squamous in appearance. An outer fibrous wall separated the cysts from the parathyroids. In the wall of one lay some strands of epithelium closely resembling that of involuted thymus. These cysts were undoubtedly thymic IV in origin but whether they arose before or after its differentiation into thymic tissue it is impossible to decide.

Cysts of type 2 were also seen in a case of thyroaplasia and congenital myxoedema in a girl of 20 months. No thyroid tissue was identified *post mortem*, and fragments of soft grey tissue from its usual site were examined microscopically. On the right side a normal parathyroid and an accidentally involuted thymus IV were found. In the thymus IV was a multilocular cyst lined with a single layer of flattened epithelium and also cysts lined with several layers of epithelium. In the tissue from the left side similar involuted thymus IV contained several cysts lined with a many-layered epithelium. Unconnected with these cysts in the few sections examined lay groups of glandular structures (Kursterner canals, type 2), the alveoli of which were lined with cubical sometimes pigmented cells while the lumina contained mucus (fig. 23). A parathyroid, like the other probably an upper, was found in the site of the left lobe of the absent thyroid. The thymus III in this subject weighed only 2 g. and was involuted and histologically

or two layers. The cyst contained a pink homogeneous coagulum which was not as hyaline as colloid, the coagulum was separated from the upper end of the cyst wall by delicate threads of mucus and included in its centre a large group of fat-phagocytes. Upon the lower part of the cyst wall lay involuted thymus tissue and it was apparent that the cyst arose from the thymus. On the surface of its upper part lay two glandular formations of considerable interest (fig 24). They were composed of vesicles or alveoli. The vesicles contained deeply eosinophil colloid material, the alveoli similar colloid or mucus. The vesicles were lined with flattened, slightly eosinophil cells. The alveoli were usually lined with cubical cells of which the cytoplasm was either eosinophil and sometimes pigmented or slightly basophil, foamy and mucous. Lining some alveoli and forming one small nodule were several large polygonal eosinophil cells with sharply defined cell membranes. These cells under a $\frac{1}{6}$ -in objective closely resembled parathyroid oxyphil cells but under a $\frac{1}{12}$ in they were not granular but minutely vesicular and did not possess pigment spheres. They were identical therefore with the large eosinophil cells found in the thyroid in certain cases of Graves' disease, Riedel's goitre and myxoedema. Some of the cells showed hyaline degeneration, which is also seen frequently in thyroid eosinophil cells. Intimately mixed with the alveoli of one glandular formation were alveoli of typical mucous gland structure, lined with cubical or short columnar cells having faintly hæmatoxyphil and finely vesicular cytoplasm. Their lumina contained mucus. Some similar mucous cells were found in the walls of the more thyroid-like alveoli. A small duct ran from this glandular formation and opened into the cyst. The glandular formations are of the nature of Kursteiner canals, resembling both the colloid vesicular type 1 and the mucous-secreting glandular type 2. The presence of a duct in one of them and their mucous alveoli show that they are not ectopic thyroid tissue. According to the records of the Bernhard Baron Institute a fourth cyst of type 3 was found in a female of 80 in 1923. Macroscopically a bunch of cysts 1.5 cm in diameter was seen 1.5 cm below the isthmus of the thyroid in front of the trachea. In the section preserved are two loculi with a wide communication. The lining is of several layers of flattened cells, in places squamous in type. Involuting thymus in the wall shows its thymic origin. In its multilocular appearance and many-layered epithelial lining this cyst resembled the thymic cysts associated with upper glands (type 2).

That these cysts may be of clinical importance is shown by a case of Dr R. D. Wright of Melbourne University, who kindly sent me the clinical details and a microscope section. A mass of about 10 g weight in the region of the bifurcation of the common carotid artery was excised from a girl of 18

PLATE XXIV

- FIG. 22 —Cyst of type 1 on surface of upper parathyroid ♂ 4 H and E. $\times 20$
- FIG. 23 —Cyst of type 2 with small gland-like Kursteiner canal (type 2) close to it ♀ 20 months H and E $\times 42$
- FIG. 24 —Glandular formation in wall of cyst of type 3 ♂ 69 H and E $\times 39$
- FIG. 25 —Cyst of type 4 on surface of lower parathyroid ♂ 54 H and E $\times 28$
- FIG. 26 —(A), group of small cystic vesicles in surface of parathyroid with large example apparently free in capsule, (B), duct like tubule and group of gland like alveoli (Kursteiner canals, type 2) in capsule near (C), a vesicle (probably Kursteiner canal, type 1) free on surface of gland and containing colloid droplets in mucous fluid ♂ 68 H and E $\times 52$

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1 per cent phosphomolybdic acid as in Mallory's method and differentiation in 0.25 per cent orange G until the cells are almost colourless while the red corpuscles still have a red tint. The inclusions are like typical cytoplasmic inclusion bodies—as seen for example in distemper and in infectious oetromelia of mice—in that their structure is mainly either uniform or spongy. They are often surrounded by a clear space and sometimes contain one or more structures with the appearance of vacuoles, being clearer than the rest of the body (figs. 1 and 2). In fig. 2 a large inclusion body in the right upper quadrant is seen to fill almost the whole liver cell with the nucleus pushed to one side. Apparently the large body has been formed by fusion of many smaller bodies. In fig. 3 one of the inclusion bodies just below the nucleus shows evidence of very fine granulation, but this is rare. The granulation usually appears to be due to larger particles and resembles a spongework rather than granules, as is demonstrated at the edge of the inclusion body in the centre of fig. 4.

When stained with hæmatoxylin and eosin the bodies stain pink, as do the red corpuscles, but of a deeper tint. With Weigert's iron hæmatoxylin and van Gieson both red corpuscles and inclusion bodies stain yellow, and occasionally fine brown granules or networks are observed in or near an inclusion body. With Giemsa (fig. 5) when the red corpuscles stain pink the inclusion bodies may retain the blue stain, some bodies being entirely blue and others pink and blue. The whole body may be blue except the vacuole, which may appear pink. Again only the edge of the body may be blue, while the centre shows a pink or faint pink and blue spongework or large granulations. In fig. 5 two blood vessels are shown packed with red corpuscles, which are easily distinguishable from the inclusion bodies.

With P. P. Laidlaw's special stain the red corpuscles are mauve and the bodies of a similar but deeper tint. Mann's method (fig. 6) stains the red corpuscles red while the inclusion bodies may be entirely of a deeper red, or part of the body may be deep blue whilst other portions are red and show the spongework. All the dark structures in fig. 6 are inclusion bodies and there are also sinus-like spaces. The liver cells have atrophied and appear like strands in this section, which may represent a later stage of the process, with greater damage to cells. Attempts to stain for iron and for amyloid in the bodies gave negative results.

Professor M. Levaditi of the Pasteur Institute, Paris, informs us that in his laboratory some inclusion bodies have been observed in the mouse's liver, and he kindly sent us some sections. In these the structures are cytoplasmic but consist of very fine granulations not very closely packed together. This condition has been passaged and appears to resemble oetromelia, but is apparently more virulent (Schoen, 1938). Attempts to passage the condition found in our mice have failed so far. It does not appear to be the same as that observed in Paris.

I am much indebted to Sir Patrick Laidlaw, F.R.S., for his kindly interest and help, also to Dr W. J. Purdy and to Mr Welch of Mr J. E. Barnard's department for assistance with the photographs.

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SCHOEN, R

1938 *C. R. Soc. biol.*, CLXVIII 695

PLATE XXV

- FIG 1 —Mouse I Relationship of inclusion bodies to liver cells P P Laidlaw's method $\times 1250$
- FIG 2 —Mouse I The large inclusion body in the right upper quadrant has been formed apparently by fusion of many small bodies P P Laidlaw's method $\times 1250$
- FIG 3 —Mouse I The liver cell in the centre shows, just below the nucleus, an inclusion body which contains a small globular mass consisting apparently of very fine granulations P P Laidlaw's method $\times 1250$
- FIG 4 —Mouse II The sponge like condition is demonstrated at the edge of the large inclusion body in the centre of the field Hæmatoxylin and eosin $\times 1250$
- FIG 5 —Mouse I The differences between the inclusion bodies and the red corpuscles are illustrated Giemsa $\times 450$
- FIG 6 —Mouse II Inclusion bodies, atrophied liver cells and sinus-like spaces Mann $\times 220$

neutralising properties in the serum, chicks were obtained whose sera also neutralised the virus completely. Sera of chicks from four mothers with no such activity were ineffective like those of their parents. When present, neutralising properties persisted in chicken sera for only a short time, as is shown in the table on p. 225.

The active immunity of newly hatched chicks was studied, but those with antibodies against the virus showed only feeble resistance to it. Falling dilutions of Rous filtrate were injected into the breast and leg muscles of the chicks. Two, the offspring of hen 9, resisted a 1:100 but not a 1:10 dilution, while four others, the young of hens with inactive sera, failed to resist the 1:100 virus. The difference was thus not very striking.

Transmission of antibodies through the egg

These results clearly suggest a transmission of antibodies through the egg, and it was in fact found that emulsions of the yolk of eggs laid by birds with antibodies had definite inactivating power against Rous filtrate, one of these eggs was from hen 9 and two from hen 38, another hen later discovered to have antibodies. The egg white had no such activity, nor was any detected in the yolk of eggs laid by a bird without antibodies. Transmission of antibodies to young chicks from an immune mother through the yolk has previously been reported in the case of tetanus antitoxin (Ramon, 1928), diphtheria antitoxin (Jukes, Fraser and Orr, 1934, Ozawa, 1936) and fowl-plague antibody (Schmidt, Oerskov and Stenberg, 1936). The neutralising property in "normal" fowl sera is thus seen to behave like antibodies in general.

Subsequent history of chicks born with antibodies

A reasonable interpretation of the finding of antibodies in "normal" fowl sera is to suppose that viruses related to that of the Rous sarcoma are widely distributed as harmless inhabitants of the fowl, frequently able to stimulate antibody production, but only exceptionally capable of causing tumours. Consideration of this idea led to speculation as to whether such a virus, like the antibody, might be transmitted through the egg. Accordingly chicks of known parentage were reared and were bled at intervals over a period of 2 years in hopes of discovering whether there was any relationship between the development of antibodies in their sera in adult life and the history of their parents in this respect. Fifty-seven sera from 41 fowls were tested. The antibodies in the sera of the parents are shown below (+ = antibodies present, - = no antibodies).

Antibodies in parents' sera	♂ + ♀ -	♂ + ♀ +	♂ - ♀ -	♂ - ♀ +	♂ ? ♀ +
No. of birds tested	4	26	5	3	3
No. of sera tested	5	41	5	3	3

Unfortunately at the end of two years none of the 41 birds had developed more than trifling amounts of antibodies in their sera and the experiment was therefore terminated. It seems unlikely, however, that development of antibodies in later life depends closely upon inherited factors. This conclusion is made more probable by the finding that a cock of Dr C. Todd's (1930) inbred stock had potent antibodies in its serum, while three siblings (2 ♂, 1 ♀) had none.

activity may be great enough to abolish the action of added complement and so make it anticomplementary in a complement fixation test in which *H v S I B* is used as hæmolsin.

Using the same technique as in the previous experiments I have examined the serum of patients treated with horse serum to see if therapeutic doses of serum produce these effects in human beings

Methods

1 *Bactericidal tests* The technique adopted was that described by Mackie and Finkelstein (1931) and consists in mixing together varying amounts of bacteria in decimal dilution and a constant amount of serum. Subinoculations were made on agar immediately, and after 4 hours' incubation. The bactericidal activity was estimated by the difference between the end-points of growth of the two series of subinoculations.

2 *Complement* Complement was estimated by adding varying amounts of fresh rabbit serum to 0.5 c.c. of a 3 per cent suspension of well washed sheep red cells sensitised by the addition of 7 M.H.D. of hæmolytic immune body. The range tested was 0.02, 0.04, 0.06, 0.08, 0.1, 0.15 and 0.2, and the activity was taken to be represented by the reciprocal of the smallest fraction of a c.c. producing complete lysis.

3 *Wassermann reaction* The Wassermann reaction was chosen as being typical of a complement fixation test. The technique adopted employed constant volumes of antigen and serum with varying amounts of guinea pig serum as complement. The amounts of complement used were 2, 4 and 8 M.H.D., with two serum controls of 2 and 4 M.H.D. The tests were carried out in duplicate, using cells sensitised with an immune body prepared in a horse and one prepared in a rabbit.

A hæmolytic immune body prepared by injecting sheep cells into a horse is referred to as "*horse versus sheep cells immune body*" or abbreviated "*H v S I B*". A similar hæmolytic immune body prepared in a rabbit is referred to as "*R v S I B*".

Results

Observations have been made upon 15 patients in the Edinburgh City Fever Hospital who had been treated with diphtheria or scarlet fever antitoxin. The first specimen of blood was withdrawn on admission before the administration of therapeutic serum and the bactericidal activity of the patient's serum upon *B. typhosus* was estimated on the same day or, at the latest, after storing till the following day in the refrigerator (4° C). One c.c. of the serum was heated to 55° C for half an hour in a quill tube and stored in the refrigerator for the Wassermann test, which was performed upon it and upon the second specimen simultaneously. The second specimen was withdrawn on the 8th-10th day after the injection of serum. It will be noted that the bactericidal tests on the two specimens of serum from any given patient were performed at different times so that it was possible for slight differences in activity to be due to the conditions of the experiment. In the Wassermann tests, however, both specimens were examined at the same time and even slight differences would have to be regarded as significant. The Wassermann test was performed in duplicate on every specimen with *H v S I B* and *R v S I B* respectively. In this test evidence was sought of the fixation of complement in the actual test and of anticomplementary action in serum control when using *H v S I B*. The results are shown in the table.

effect in a complement fixation test using a hæmolytic immune body prepared in a horse

I wish to thank Dr A Joe of the City of Edinburgh Infectious Diseases Hospital for the specimens of blood from his hospital

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Portrait by

[Hay Wrightson, London, W 1

LEONARD STANLEY DUDGEON

the laboratory was it was built on the main corridor of the first floor and in the very middle of the hospital, and under Dudgeon's guidance it became the centre of hospital life. The staff consisted of a director, an assistant director and a boy, but senior students were allowed to help in the work of the little department. For a clerkship in the clinical laboratory there was always keen competition and successful candidates for this appointment thought themselves to be, and indeed were, fortunate among their fellows. As the clock struck ten a firm and familiar footstep was heard in the corridor, the door of the laboratory was thrown open, a silk hat was hung on a peg and a very large overall was put on: the day's work had begun. The examination of every specimen which came to the laboratory was as thorough and complete as the methods of the day allowed. A high standard of technical skill was expected. Clumsiness was rebuked with jovial abuse but carelessness and slackness were sins for which there was no forgiveness. Every specimen was submitted to long and careful scrutiny and the results of the examination were embodied in a written report which was short, definite and final. Many of Dudgeon's pupils acquired in a few months considerable manual dexterity and a useful knowledge of laboratory methods. There can have been few who failed to appreciate the importance of care, accuracy and honesty in the application of laboratory methods to clinical practice. The visits which Dudgeon paid to cases in the wards to collect specimens or to confer with clinical colleagues provided fruitful opportunities for instruction. Dudgeon never collected a specimen for a laboratory test until he had made himself familiar with the details of the case. Usually he examined the patient. In these short lessons on bedside pathology Dudgeon rendered his pupils his greatest service. From this repeated correlation of the results of clinical observation with laboratory findings he built up an experience, probably unrivalled, which formed the basis of his outlook on pathology and influenced all his teaching.

Not the least valuable of Dudgeon's qualities was his great energy, which enabled him to work long hours on end without loss of interest and in his earlier days without signs of fatigue. The routine work of the clinical laboratory occupied him continuously from ten o'clock until teatime. He ate no lunch on week days. At five o'clock he began his research work which was often continued with an interval for dinner until a late hour of the night.

During the period which began with his appointment as superintendent of the clinical laboratory and which ended with the outbreak of the War, Dudgeon occupied a very important position in the life of St Thomas's Hospital and exercised a remarkable influence on its medical school. He earned and held the respect of his senior colleagues and made the clinical laboratory one of the

ideal advisor for harassed medical officers faced with practical difficulties that would not brook delay. And his advice was not only practical, it was good, for his opinions, though didactic in form, were always based on accurate observation and a careful consideration of all relevant factors.

Dudgeon returned to St Thomas's in 1919 and in July of that year the University of London conferred on him the title of professor of pathology. He became increasingly absorbed in administrative duties and on the retirement of Sir Cuthbert Wallace, he succeeded to the deanship of the Medical School. He took over a flourishing institution and under his rule the School never looked back. Owing to the generosity of certain of his private patients and to his own financial ability, he was able to carry out many necessary structural alterations, rebuilding the anatomical department and the post-mortem rooms. Though himself essentially practical in outlook, he was always in sympathy with the teachers of the scientific subjects and lent a ready ear to their requests for improved equipment. He realised that the London student had suffered in the past from a relatively indifferent grounding in the basic sciences as compared with the man who joined the School from the older universities, and to obviate this defect he developed a system under which selected students were enabled to proceed to a B.Sc. degree before passing on to clinical subjects. He also did much to establish a liaison between the hospitals of the London County Council and the teaching schools, with a view to rendering the vast clinical material of the public institutions available for the instruction of the medical student. He served on the Voluntary Hospitals Committee and was chairman of the Deans Committee and a member of the Senate of the University of London. With these numerous administrative duties and the increasing demands of private practice, Dudgeon during his later years had but little time available for research. After his return from Macedonia he published some valuable papers on the dysenteries and latterly developed a rapid method of tumour diagnosis by smears, which he applied with conspicuous success to sputum, gastric contents and excised growths. He was an excellent teacher and possessed a fund of information derived from forty years' experience of clinical pathology which was always at the disposal of his younger colleagues.

On the teaching of pathology, especially in the London schools, Dudgeon's influence was considerable. He taught pathology not as an isolated subject but as an integral and essential part of medical science. In all his teaching he emphasised the close relationship of pathology with clinical medicine and surgery but he never allowed his pupils or his colleagues to forget that a sound knowledge of pathology was as essential as clinical experience in

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Dudgeon returned to St Thomas's in 1919 and in July of that year the University of London conferred on him the title of professor of pathology. He became increasingly absorbed in administrative duties and on the retirement of Sir Cuthbert Wallace, he succeeded to the deanship of the Medical School. He took over a flourishing institution and under his rule the School never looked back. Owing to the generosity of certain of his private patients and to his own financial ability, he was able to carry out many necessary structural alterations, rebuilding the anatomical department and the post-mortem rooms. Though himself essentially practical in outlook, he was always in sympathy with the teachers of the scientific subjects and lent a ready ear to their requests for improved equipment. He realised that the London student had suffered in the past from a relatively indifferent grounding in the basic sciences as compared with the man who joined the School from the older universities, and to obviate this defect he developed a system under which selected students were enabled to proceed to a B Sc degree before passing on to clinical subjects. He also did much to establish a liaison between the hospitals of the London County Council and the teaching schools, with a view to rendering the vast clinical material of the public institutions available for the instruction of the medical student. He served on the Voluntary Hospitals Committee and was chairman of the Deans Committee and a member of the Senate of the University of London. With these numerous administrative duties and the increasing demands of private practice, Dudgeon during his later years had but little time available for research. After his return from Macedonia he published some valuable papers on the dysenteries and latterly developed a rapid method of tumour diagnosis by smears, which he applied with conspicuous success to sputum, gastric contents and excised growths. He was an excellent teacher and possessed a fund of information derived from forty years' experience of clinical pathology which was always at the disposal of his younger colleagues.

On the teaching of pathology, especially in the London schools, Dudgeon's influence was considerable. He taught pathology not as an isolated subject but as an integral and essential part of medical science. In all his teaching he emphasised the close relationship of pathology with clinical medicine and surgery but he never allowed his pupils or his colleagues to forget that a sound knowledge of pathology was as essential as clinical experience in

may be applied to the vessel wall " In arteriosclerosis the exudative response appears to be most often either serofibrinous or hæmorrhagic In the arterial wall exudates are of little significance unless they are massive and cannot be removed promptly They may then remain with or without the addition of dead elements of the tissues to act as stimuli for subsequent organisation In this connection it was found that proliferation of capillary endothelium was as often predominant as was connective tissue proliferation, a fact to which very great importance is attached Evidence is adduced to show that by the formation of intramural collateral channels in cases of arterial occlusion or constriction a considerable amount of blood may be "shunted" round the occluded or constricted region The cross section of such an accessory bed is sometimes considerable, and the significance of a collateral circulation of this type may be very great in regions where other routes are either not available or insufficient in capacity, as in the so-called end-arteries of the coronary system On the other hand, if the agent primarily responsible for initiating the vascular lesion should persist, or a succession of such agents should impose themselves upon the vascular tissue, the greatly exaggerated vascular bed would certainly enhance any tendency to oxudation, and, being easily damaged, might also be a source of hæmorrhage into what would otherwise be a lesion of little consequence

"Proliferation of mononuclear phagocytes is a prominent feature in many sclerotic lesions . . The ability to segregate lipoid materials from any available source is one of the remarkable attributes of these cells, and large numbers of them filled with fat may gather in the intima, in the media, and even in the adventitia The lipoid present in the arterial lesions may be derived from the serum lipoids, or from the débris of cells and exudate that follows upon necrosis of some portion of the vessel wall "

The importance of focal intramural hæmorrhage in the histogenesis of arteriosclerosis is frequently mentioned Its ætiology is varied, the hæmorrhagic diatheses, vitamin deficiencies, conditions which increase the permeability of the capillary wall, and various mechanical, chemical and parasitic agencies are referred to

The authors have also investigated the vascular supply of the heart valves, and have found the number of injected vessels to vary tremendously A simple vascular pattern similar to that found regularly in the cow was present in some normal valves, in others no vessels whatever were seen The so-called normal pattern consists of vascular arches derived from vessels at the myocardial attachment of the valve Occasionally secondary networks arising from these arches extend a little further into the valve In the auriculo-ventricular valves a hitherto undescribed source of vascular supply has been discovered Small vessels were found entering the body of the valve directly from the endocardial surface By contrast with the healthy organ, diseased valves exhibit tremendous vascularity "In cases of rheumatic endocarditis, sub-acute bacterial endocarditis, and syphilitic involvement of the aortic valve, the injected and cleared specimens show dense arborizations of interlacing vessels of small caliber, chiefly capillaries Associated with these are hæmorrhages, focal areas of calcification, both within and without the lumina of dilated sinusoids, as well as exudations of fibrin and leucocytes In other cases less fulminating in character, and not associated with definite etiological agents the valves often resemble sclerotic vessels in appearance " In general

enter without any previous instruction in this subject, and dealing later with vehicles of infection, clinical bacteriology and mycology, immunity, public health laboratory work, public health field and office work and parasitology. Its composite nature differentiates it somewhat from other publications of this sort and may make the book of interest to teachers in this country. Each section consists of a brief introductory account of the work undertaken and a group of illustrative exercises which follow familiar lines. Much of it is difficult to read without reference to local practice and nomenclature and it is on the whole sketchy. The statement (p. 36) that "bacteria have the walled cells of a plant, and are usually included among the fungi, as they require organic food and lack chlorophyll" is a generalisation which cannot be substantiated, nor can the statement that flagellar motility and antigenic property are reduced or lost in the change from smooth to rough (p. 59). The views expressed on the nature of bacteriophage (p. 79) are not in agreement with recent work. No mention is made of the use of tellurite or of type differentiation in the section on diphtheria. The reference on p. 232 to anaphylatoxin is misleading, as is the statement on p. 251 that the agglutinin in a bacterium is a protein. Some sort of reference to antigen analysis might have been made in the section on food poisoning. Illustrations of plate pouring, tube plugging and filter paper folding and uncoloured illustrations of pigment production or the reductase test are surely either unnecessary or useless and if the theories of immunity are to be discussed and illustrated this ought to be done with reference to recent work and not simply illustrated in the old Ehrlichian way. The publishers show their optimism regarding the circulation of this volume by labelling the wrapper "First edition."

Bacteriology for medical students and practitioners

By A. D. GARDNER. Second edition. London. Humphrey Milford, Oxford University Press. 1938. Pp. 274, 32 text figs. 6s.

The appearance of the second edition of this little book indicates that it has attained a certain popularity among students. It continues to be a miracle of compression with little to stimulate further study except a hunt in the new preface. There are remarkably few errors, though the compression effected leads here and there to slight ambiguities.

Synopsis of clinical laboratory methods

By W. E. BRAY. Second edition. London. Henry Kimpton. 1938. Pp. 408, 51 text figs. and 17 colour plates. 18s.

By adding nearly a hundred pages to the text, the author has been able to include a considerable number of additional procedures in the new edition of his book. He has also increased the number of text illustrations and colour plates. But the original conception of a summary of procedures has been kept and the book is a mine of information in a condensed form. The criticism offered to the first edition still holds good, that brevity in the description of the method of performing tests may lead to the omission of necessary practical details.

That a second edition should have been called for within two years speaks clearly for the fact that the book has been found of value. The accuracy of the subject-matter must largely account for this.

- C L. OAKLEY and G. H. WARRACK. Persistence of influenzal antibody in mice
- A S McFARLANE Properties of vaccinia virus
- C H STUART-HARRIS A neurotropic strain of human influenza virus
- JANET S F NIVEN (1) Neuro-epithelial cyst of the third ventricle. (2) Tumour of the thymus
- B G MAEGRAITH and H M CARLETON Aortic arteriosclerosis in rabbits
- R KNOX A circular manifold for the desiccation of tissues and other biological materials
- G J CUNNINGHAM and C REBURN A case of sympathogonioma of the left adrenal
- S L BAKER (1) A freezing method for making sliced museum specimens of bone and soft tissues (2) Three tumours involving the tarsal bones - (i) chondrosarcoma, (ii) sarcoma of tendon sheath origin, (iii) slow-growing epidermal carcinoma (3) An unusual type of reticulum-cell sarcoma
- *H BURROWS Intersexuality induced in female rats by testosterone
- H A MAGNUS Two cases of reticulosis exhibiting a marked degree of erythrophagocytosis
- C ROBINOW Nucleoid bodies in bacteria as demonstrated by the Feulgen method
- R D. WRIGHT and H W FLOREY. The influence of nerves and hormones on the gastro-intestinal tract (cinematographic demonstration).
- A G SHERA New type of electric saw for use in the post-mortem room.

Abstract.

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INTERSEXUALITY INDUCED IN FEMALE RATS
BY TESTOSTERONE

HAROLD BURROWS

Research Institute, Royal Cancer Hospital (Free), London

The development in the female, under treatment by testosterone, of external genitalia resembling those of the male has been already recorded by Raynaud (1938 *a* and *b*) in mice, Dantchakoff (1938) in guinea-pigs, and Hamilton and Gardner (1937-38) in rats. The phenomenon as occurring in nature has long been recognised in freemartins, and has been reproduced experimentally by grafting testes into castrated litter-mate females. The rats now shown represent a litter of 8 born on the 9th or 10th of November last. There are 5 females and 3 males. Within 24 hours of birth they were each given a subcutaneous injection of 60 γ testosterone in sesame oil. The doses were repeated on the following day and thereafter three times a week. On 21st November the amounts of testosterone were increased to 150 γ and later to 300 γ , which is the dose now being given. In the females the external genitalia are modified. In four there is no vaginal opening, and in the fifth the opening is minute. In all five a well-developed penis is present with extensive preputium and a glans furnished in some instances and probably in all with an os penis. In one of the females priapism has persisted for two weeks.

(1931), Kant (1932-33), Bender and Schilder (1933) and others. Gamper's description of the pathological anatomy can hardly be improved on, though minor additions have been made. He noted the curiously selective distribution of focal lesions throughout the grey matter of the brain stem, showing hyperæmia (sometimes but not necessarily with small hæmorrhages), vascular proliferation (endothelial and mesenchymal), a varying degree of glial proliferation, absence of inflammatory infiltration and relatively slight evidence of damage to the nerve cells. The localisation of the lesions was remarkably characteristic, they were constantly and most markedly seen in the corpora mamillaria, less constantly in the other hypothalamic nuclei and in the nucleus parafascicularis and medial part of the medial nucleus of the thalamus, in the mid-brain they were frequently seen in the periaqueductal grey matter (involving the oculomotor nuclei), in the posterior colliculi and in the central grey matter at the junction of the mesencephalon and diencephalon, and in the hind-brain in the grey matter of the floor of the fourth ventricle, especially affecting the dorsal vagal nuclei and the anterior end of the eminentia teres, *i.e.* the lesions extended from the medulla forwards to the anterior commissure. Nothing characteristic was found in the cerebral cortex. Gamper associated the lesions in the corpora mamillaria causally with the Korsakow's psychosis which was seen clinically in his cases.

Though Wernicke's original three cases included one of non-alcoholic origin and though from time to time other non-alcoholic cases have been reported, alcohol was regarded by Gamper and later authors as the important ætiological agent. Neuburger, however, has recently (1936-37, 1937-38) emphasised the incidence of the condition in non-alcoholics as a complication of various diseases and concludes that it occurs much more commonly than has hitherto been realised. He has reported a series of cases occurring in individuals suffering from carcinoma—especially gastric carcinoma (1936-37), and chronic gastritis (1937-38). He points out that in such patients, dying perhaps from a disease recognised as beyond the reach of treatment, the onset of cerebral symptoms may easily be overlooked or neglected, and that in the examination of the brain, the corpora mamillaria, the most constant and sometimes the only site of the lesions, may easily escape examination.

Recent experience has convinced us of the truth of Neuburger's statements, and the following series of twelve cases of Wernicke's encephalopathy seems to us to be worth recording, firstly because of the twelve only one was associated with alcoholism, while in most of the others alcoholism can be definitely excluded, secondly because the incidence of our cases as complications of a variety of different diseases may throw some light on the pathogenesis of the condition, and thirdly because the scantiness of references to Wernicke's encephalopathy in the British literature combined with our experience of it as a not very rare occurrence leads us to believe that it is frequently missed both clinically and at autopsy in this country.

Source of cases

Table I shows the various conditions with which we have found Wernicke's encephalopathy associated. Our cases were derived from general, maternity and fever hospitals, but since,

The pathology of Wernicke's encephalopathy

Autopsy was performed on each of the twelve cases. For histological examination the following methods were employed

Paraffin sections were stained with hæmatoxylin and eosin, by Nissl's method (cresyl violet) and by Foot's method for reticulin, thick frozen sections were stained by Pickworth's benzidine method for demonstration of the vascular pattern (Pickworth, 1934-35), and thin frozen sections with Scharlach R and hæmatoxylin for fat, by Cajal's gold chloride and Anderson's Victoria blue for astrocytes and by Globus' modification of Del Río-Hortega's silver carbonate for microglia

In some of our cases Wernicke's encephalopathy was an unexpected finding in the course of routine examination of the brain, this was so in cases where the patient was admitted *in extremis*, with a history in which the primary disease (*e.g.* gastric carcinoma) bulked largely, and where a satisfactory examination of the nervous system could not be made. In most cases, however, the clinical history pointed to a cerebral complication of the primary disease and in some cases Wernicke's encephalopathy was diagnosed before death. It is possible, therefore, especially in the presence of certain primary diseases, to open the skull in reasonably confident expectation of finding the characteristic cerebral lesions.

Externally, as a rule, the brain shows nothing abnormal. Rarely the corpora mamillaria may show obvious congestion externally. The leptomeninges and the pial vessels show nothing of note.

On section of the brain the diagnosis is, in most cases, at once obvious. There may or may not be a moderate degree of generalised congestion, but in the typical case (figs 1-5) foci of much more marked congestion with many small petechial hæmorrhages are seen, which selectively affect certain regions. The lesions are almost without exception symmetrical. The corpora mamillaria are constantly involved, and frequently also a zone of congestion with petechiæ is seen in the grey matter immediately surrounding the third ventricle, *i.e.* throughout the hypothalamus and medial part of the thalamus on each side. This periventricular zonal lesion may be continuous downwards with a similar zone of congestion and petechiæ in the grey matter surrounding the aqueduct, or small discrete focal lesions may be seen in the aqueductal grey matter. Foci are also frequently seen in the posterior colliculi of the mid-brain and, less frequently, in the grey matter of the floor of the fourth ventricle and other regions (table II).

In the typical case, showing involvement of most of these

regions, the naked-eye picture is striking and pathognomonic. In some, however, the distribution of lesions is much more limited. They may be found in the corpora mamillaria and practically nowhere else (*e g*, cases 6, 7 and 12), and may therefore easily be missed if the brain is not sectioned carefully and the main sites of predilection (particularly the corpora mamillaria) examined. Furthermore, even where lesions are present they are not always obvious to the naked eye, petechiæ are not always visible—indeed in the corpora mamillaria, the most constant site of the lesions, macroscopically visible hæmorrhages are in our experience more often absent—and even congestion may not be striking. In cases 6 and 9 the focal congestion was so slight and the petechiæ so small in the affected sites that had the diagnosis not been suggested from the clinical history it would have easily been missed on macroscopic examination. As Neuburger emphasises, histological examination of the corpora mamillaria is the essential diagnostic step. Almost always the lesions are symmetrical, but case 2, in which a lesion was found only in one corpus, shows that sections should be made of both corpora in a doubtful case.

Table II shows the distribution of the lesions in our twelve cases. Not all the possible sites were examined histologically in every case, and small focal lesions may easily have been missed, in case 6, for example, the clinically noted ophthalmoplegia (table I) indicated a focus in the mid-brain which we did not find on examining the brain (serial sections of the mid-brain were not made). The order of frequency of affection of the sites of predilection which we give in table II is therefore not to be taken as exact. It corresponds fairly closely, however, with that noted by Gamper, Neuburger and others. No significant lesions were found in the cerebral cortex in any of our cases.

Histology

Histologically the lesions in our cases are constant in type, though varying considerably in intensity and age. They agree closely with the description given by Gamper and later authors of Wernicke's encephalopathy of alcoholic origin. Briefly they consist of foci, well defined in some sites (*e g* the corpora mamillaria), more diffuse in other sites, showing evidences of vascular disturbance, parenchymatous degeneration and glial reaction. The vascular disturbances consist of great dilatation of supracapillaries and some capillaries—other capillaries being anæmic (probably because of endothelial swelling), irregularities of calibre and bead-like dilatations of these vessels and, to a very varying degree, small perivascular (presumably diapedetic) hæmorrhages both of "ball" and "ring" type (figs 6-10) this picture, well shown by

or merely a slight swelling of nucleus and cytoplasm without increase in numbers. Subacute cases show (fig 15) both swelling and proliferation, with formation of moderate numbers of glial fibrils.

The lesions contrast sharply with those of the infective forms of encephalitis in that there is little or no evidence of leucocytic emigration. In the more acute hæmorrhagic Wernicke lesions an occasional emigrated polymorph may be seen and an occasional case may show infiltration of the perivascular sheaths with lymphocytes, but these findings are exceptional. Luthy and Walthard (1928) suggest that lymphocytic infiltration where present is a secondary phenomenon due to metastatic infection of the already existing lesions from some distant infective focus, but they offer no bacteriological proof of this. Only three of our cases (nos 2, 5 and 12) showed any perivascular lymphocytic infiltration, in all three it was slight and in two it was found not in the Wernicke lesions themselves but in other parts of the brain. We can offer no explanation of its presence.

The condition was originally called by Wernicke a "polio-encephalitis". Undoubtedly the lesions affect grey matter predominantly, but as several authors have pointed out, there is often a spread to adjacent white matter—notably the columns of the fornix, which may show petechiæ right from their junction with the corpora mamillaria up to their fusion to become the body of the fornix. Further evidence of involvement of white matter is seen in the foci in the optic nerves in our case 11, where a small focus was found near the middle of each optic nerve, showing compound granular corpuscles in the connective tissue septa and granules of fat, partly free and partly in phagocytic cells, among the nerve fibres. Spielmeyer's stain showed no obvious demyelination. There was a slight vascular endothelial hyperplasia in and around this focus, but no hæmorrhages were seen.

We have not found previous record of involvement of the optic nerves in Wernicke's encephalopathy diagnosed as such, but several authors have recorded the occurrence of retinal hæmorrhages (non-hypertensive) in hyperemesis gravidarum and polyneuritis gravidarum (Albeck, 1922, Berkwitz and Lufkin, 1932, Stander, 1932, Tillman, 1934; and Randall and Wagener, 1937). The last two of these references cite cases where the brain showed changes very suggestive of Wernicke's encephalopathy, though not identified as such by the authors. This, combined with our finding of focal degeneration in the optic nerves in our case 11, suggests that the retina and the optic nerves may be included among the vulnerable areas in this disease. It will be remembered that the anterior colliculi are sometimes affected (as they were in case 11), thus apparent common vulnerability of several anatomically remote

in head injury and, apparently as a terminal phenomenon, in other conditions may at first sight appear similar, but in such cases the other histological phenomena of the Wernicke lesion are absent, and the corpora mamillaria are not affected. A "hæmorrhagic encephalitis" is sometimes found in cases of head injury, sepsis, carbon monoxide and arsphenamine intoxication, etc., but this "encephalitis" consists of petechial hæmorrhages throughout the cerebral white matter, with no tendency to attack the Wernicke sites. The same applies to the apparently primary hæmorrhagic encephalitis described by Baker (1935). The acute toxic encephalitis described by Grinker and Stone (1928) in children with various acute infections shows congestion, vascular endothelial hyperplasia and degenerative changes in nerve cells, but the changes are diffuse and histologically quite unlike those of the Wernicke lesions, microglial reaction is absent, for instance. Lastly the infective forms of encephalitis are sharply distinguished by the prominence of lymphocytic or other leucocytic emigration, which as we have seen is exceptional and slight in Wernicke's encephalopathy.

Correlation of the pathological anatomy with the clinical picture

In table I the clinical features of our cases are summarised. Detailed descriptions would be out of place here. Good clinical descriptions of the alcoholic form of Wernicke's encephalopathy have been given by Kant (1932-33) and Bumke and Kant (1936). Briefly, the picture is usually one of lethargy or drowsiness terminating in coma, sometimes with periods of excitement, exceptions to this picture were shown, however, by our case 2, which showed mental confusion and periods of excitement without obvious drowsiness, and case 12, which showed continuous excitement. Focal signs are usually present, varying according to the distribution of the lesions. Eye signs—nystagmus, ophthalmoplegias of various kinds, Argyll-Robertson pupil—are common, as one would expect in view of the frequency of mid-brain lesions. In case 11 the bilateral central scotoma is explained by the focal lesions in the optic nerves. Symptoms due to the lesions in the vegetative centres of hypothalamus and floor of fourth ventricle are also seen, among our cases, for instance, glycosuria was noted twice, and irregularities in respiration or death predominantly from respiratory failure also twice. Vomiting was a very frequent symptom, but in cases such as ours it is difficult to say whether it was due to the primary disease which caused the encephalopathy or to the focal lesions in the hypothalamus or medulla.

When the drowsiness was not too severe to make assessment of mentality impossible, some of our cases (*e.g.* nos 1, 2 and 5)

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seven such cases, Elkington (1936-37) has reported a case of Wernicke's encephalopathy in which the clinical picture seems to have suggested gastric carcinoma, though no autopsy record of the visceral findings was given, Shimoda and Yamashita (1934-35) record a case following gastrectomy for gastric carcinoma, and three of our own twelve cases had gastric carcinoma.

A further group of cases is associated with other disturbances of the alimentary tract. Neuburger (1936-37, 1937-38) records three cases associated with chronic gastritis, one with melanoma showing metastases in stomach and liver, and one with carcinoma of the rectum. One of Wernicke's original cases followed sulphuric acid poisoning, but the symptoms of the cerebral lesions came on after an interval, and at autopsy a pyloric stenosis was found. One of Shimoda and Yamashita's cases followed gastro-enterostomy for chronic peptic ulcer. Among our cases one had an old gastro-enterostomy and had suffered from repeated gall-stone colic, one had had an ileo-cæcal resection and showed a macrocytic anæmia suggesting deficient alimentary function, at least as regards the absorption of a hæmopoietic factor or factors and one had pernicious anæmia with its associated gastric dysfunction. Gaupp (1937) records a case which followed cholecystectomy for gall-stone colic, there is no note of any actual lesion of the gastro-intestinal tract, but the operation was followed by a good deal of vomiting. Uchimura and Akimoto (1935) record a case associated with cerebral vascular syphilis, but their case also had a long history of gastro-intestinal symptoms and showed chronic interstitial hepatitis at autopsy.

Another group of cases is associated with pregnancy and is brought on usually (perhaps always) by hyperemesis gravidarum. We have recorded two such cases, one with definite hyperemesis, the other with a vaguer condition of great flatulence and some vomiting, though hardly enough to indicate a typical hyperemesis. The association of Korsakow's psychosis with pregnancy and hyperemesis gravidarum has of course long been known, and since, as we have seen, the evidence is in favour of Wernicke's encephalopathy being a common if not the invariable pathological basis of this psychosis in alcoholics, one would expect to find the same encephalopathy in the pregnancy cases. We have not actually found any such cases in the literature where a Wernicke's encephalopathy was recognised, but among accounts of psychoses of pregnancy, polyneuritis of pregnancy, and a heterogeneous group of cerebral complications known as cerebropathia toxica gravidarum, we have found cases recorded where the brain showed changes very suggestive of Wernicke's encephalopathy, though not recognised as such (Berkwitz and Lufkin, 1932, Naujoks and Uffenorde, 1932, Tillman, 1934). Wernicke's encephalopathy has

either in location (restricted to the corpus striatum) or in histological appearance. Our cases certainly illustrate the frequency of gastrointestinal disturbance in Wernicke's encephalopathy (gastric carcinoma, gastric defect of pernicious anæmia, old gastro-enterostomy, ileo-cæcal resection), but we did not find liver damage a striking feature, fatty change was frequently present but was rarely severe. We do not feel, therefore, that Neuburger's emphasis on the endogenous toxin theory is justified although such a toxin may play a part, it seems unlikely to be the major factor, especially as no such cerebropathic toxin of endogenous origin has as yet been shown capable of reproducing lesions with a close resemblance to those of the human disease.

Anæmia does not seem to be a factor of major importance as far as our cases are concerned, some of them showed a moderate degree of anæmia, but in no case was it severe, not even in the case of pernicious anæmia, and the encephalopathy has not to our knowledge been reported as a complication of really severe anæmias.

Vitamin deficiency was suggested as a possible factor in alcoholic Wernicke's encephalopathy by Bender and Schilder but was not stressed. Neuburger (1937-38) also discusses it, but, we feel, underrates it. Impressive evidence can be adduced especially for deficiency of vitamin B₁. In chronic alcoholism one finds a frequent clinical-pathological triad in Korsakow's psychosis, polyneuritis and Wernicke's encephalopathy. Within the last few years the belief has grown that the polyneuritis of alcoholism is analogous to that of beri-beri and is due to a B₁ deficiency, the work of Strauss (1935) and Jolliffe, Colbert and Joffe (1936) has, we believe, established this beyond doubt.

Strauss showed that the polyneuritis can be rapidly improved by B₁ therapy and that this improvement can be produced despite the continued administration of large amounts of alcohol, Jolliffe and his co-workers showed that the vitamin B₁ intake of patients with alcoholic polyneuritis was consistently below the necessary minimum (based on Cowgill's formula), while that of a group of chronic alcoholics without neuritis was above it.

This at once suggests that B₁ deficiency may enter into the causation of the other members of the triad, Korsakow's psychosis and Wernicke's encephalopathy.

We find the same triad associated with pregnancy, especially (if not always) with hyperemesis (see page 256). And here again there is increasing evidence (Wechsler, 1933, Strauss and McDonald, 1933; Strauss, 1936) that deficiency of vitamins, particularly B₁, is responsible for the polyneuritis. In favour of this Strauss cites the persistent vomiting with resulting poor food intake, the tendency to treat hyperemesis with a high carbohydrate diet which increases the demand for B₁, the rise in metabolic rate during pregnancy which has the same effect, the gastric hypo-

the symptoms, and Sato believes (Takamatsu and Sato, 1934) that the pathological mechanism of the condition is a combination of avitaminosis B and some unidentified toxic factor. Here again, therefore, there is some evidence of the participation of avitaminosis B in the production of Wernicke's encephalopathy.

Experimental work gives further support to this view. The more or less diffuse degenerative changes in the white matter of the cord and in the peripheral nerves which have been reported in experimental deficiency of vitamins A, B₁ and B₂ by Mellanby, Zimmerman and his co-workers and others do not concern us here, these lesions are quite distinct from those of Wernicke's encephalopathy. Prickett, however (1934), showed that rats on a diet deficient in B₁—or at any rate in the heat-labile part of the B complex—developed foci of congestion, hæmorrhage and parenchymatous degeneration with formation of compound granular corpuscles in pons, medulla and cerebellum. He did not identify these with the human disease. Alexander, Pijoan and Myerson (1938) have recently reported hæmorrhagic lesions in the basal ganglia and brain stem of pigeons on a B₁-deficient diet which they state to be very similar to those of Wernicke's encephalopathy in man. These lesions were not produced by diets deficient in B₂, C or the fat-soluble vitamins.

This is not to say that in the human disease B₁ deficiency alone is responsible for the lesions, indeed this seems unlikely, since Wernicke's encephalopathy does not seem to have been found in beri-beri, which is probably the most clear-cut B₁ avitaminosis in man. Possibly a multiple deficiency is responsible, possibly B₁ deficiency acts in conjunction with an endogenous toxin such as Neuburger postulates. Peters (1936) and his school have shown that in B₁-deficient pigeons there is disturbance of carbohydrate metabolism in the brain, especially in the lower parts of the brain, resulting in accumulation of lactate and appearance of pyruvate. Peters does not believe that these carbohydrate metabolites are themselves toxic, but presuming that a similar process occurs in the B₁-deficient human brain, one may well postulate that the resulting abnormal metabolic conditions may predispose certain areas (*i.e.* corpora mamillaria, etc.) to the action of a superadded toxin or the superadded deficiency of another vitamin.

It must be admitted that in a small heterogeneous group of cases of Wernicke's encephalopathy there is nothing to suggest either poor intake or poor absorption of vitamin B₁ or other vitamins. Our cases 10, 11 and 12 fall into this group. In cases 10 and 11 the apparent primary disease was infective—bronchiectasis in one and pyosalpinx in the other. In case 12 the clinical and pathological diagnoses were never satisfactorily established, whooping cough was considered a possibility but was never confirmed. This case

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strain yielded a percentage incidence of skin cancer not higher than that in mice with a low mammary cancer incidence. Evidence of antagonism in the development of carcinoma in the two sites, skin and breast, was also obtained (1) There was a retardation in the onset of skin cancer in the females of the RIII strain as compared with the males, that is, at the 30th week six out of twelve males had already developed tar cancer whereas none of 14 females had developed it, although four females had already developed breast cancer At the end of the experiment, 56 weeks after the beginning of tar painting, the total number of tar cancers was the same for both sexes (2) Of 14 RIII females shown in Cramer's chart 2, six eventually developed mammary cancer, two of these developed no skin tumour but died early, two developed a tar wart, one of which was in existence for a very long time but failed to become malignant, and two developed tar carcinoma Of the eight females which did not develop mammary cancer, six developed tar cancer and two remained completely negative (3) There was some indication that the incidence of mammary cancer was lower in tarred than in non-tarred females of this strain

As these results did not at first sight appear to accord with those of our own earlier experiment it was decided to reinvestigate the question A further analysis of the experiment already recorded is made here and in addition the results of a new experiment are given

Description of the female mice of the white label (Kreyberg) strain.

The litter received from Dr Kreyberg, to whom our grateful thanks are due, comprised eight females and three males and was called generation 1 By means of strict and unselected brother-sister mating, seven filial generations have been obtained In experiment I, mice from generations 1-4 were used in experiment II, mice from generations 5-8

It was soon noticed that a considerable number of the females developed spontaneous mammary cancer, including three of the original eight mice received from Dr Kreyberg In the first eight generations bred in this laboratory, 332 female mice attained the age of seven months—the time of appearance of the first mammary cancer This group comprised 147 mice which were not treated, 162 which were painted with tar and 23 which received an intraperitoneal injection of 1 2 5 6-dibenzanthracene Of these 332 mice, 90 developed cancer of the breast, an incidence of 27.1 per cent This approximates very closely to the incidence of this type of cancer in this strain in Norway (Kreyberg, 1935), where of 475 females 122 developed breast cancer, an incidence of 25.7 per cent But Kreyberg reported a marked segregation in two lines, one line giving a very high incidence of spontaneous breast cancer, the other line developing no breast cancer at all This has not been observed in the eight generations in this laboratory, the tumours appearing to develop at random and no line being entirely free

Development of skin cancer and breast cancer in tarred mice

Experiment I Details of technique are given in a previous paper (Bonser, 1938). Briefly, an ether extract of a highly carcinogenic tar was applied once weekly to the skin of the back between the shoulder blades for 29 weeks. Observations were made of the appearance of tar warts, malignant change in these warts as estimated by palpation and subsequent histological examination and breast cancer. No attempt was made to excise the tumours as the experiment was designed to show differences in tar response between this and other strains. At the end of the 29th week of tarring all the living animals were killed and a histological examination was made of the skin with its tumours.

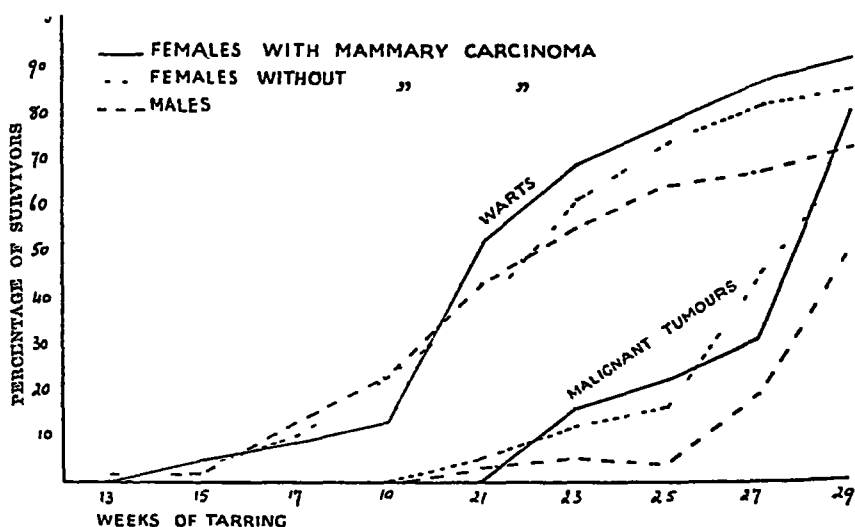


CHART 1—Experiment I Development of warts and malignant tumours of skin in tarred animals (1) percentage of warts in survivors, (2) percentage of malignant tumours in survivors with warts

and of the breast cancers as well as of tissues from mice which died before this date

The total number of animals alive at the end of the 13th week of tarring, that is the date at which the first wart appeared, was 160. These will be considered in three groups—30 females which developed mammary carcinoma, 55 females which were free from mammary carcinoma and 75 males. The mortality in the first group was considerable, 12 mice dying between the 13th and 28th week of tarring with breast cancer but without warts, in the other two groups it was negligible. Allowance is made for deaths occurring during the period of the experiment by reckoning the wart and tar cancer incidence in relation to survivors. Chart 1 shows the development of warts in the survivors of all groups

that the question was worthy of further investigation and therefore another experiment was undertaken, in which the conditions of the treated and untreated groups were very carefully controlled

Experiment II White label mice were bred by brother-sister mating and the females of each litter were divided into two groups, each of which was kept under identical conditions. All were virgins. One group of 69 mice received no treatment and was observed until death; in the other group (73 mice) tarring was commenced at 12 weeks of age and continued weekly for 24 weeks. The mice were then observed. No attempt was made to excise either the breast tumours or the tar tumours, as it was thought advisable to allow any antagonistic action of the one for the other to be present for as long as possible.

One mouse in each group died before the age of seven months and was discarded. Of the 72 tarred mice, only seven developed breast cancer. A comparison, therefore, of the effect of the presence of a breast cancer upon the development of warts and tar cancer is of less value than in expt I, as the number of mice with breast cancers is much smaller. From chart 2 it is seen

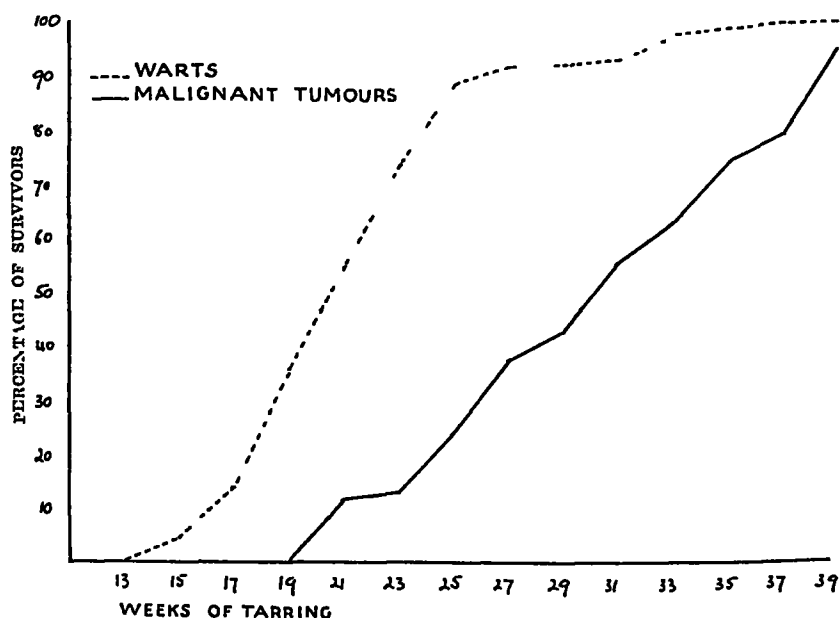


CHART 2—Experiment II Development of warts and malignant tumours of skin in tarred animals without breast cancer (1) percentage of warts in survivors, (2) percentage of malignant tumours in survivors with warts

that wart development in the 65 mice without breast cancer proceeded in the same way as in expt I, 50 per cent of survivors have warts at 20½ weeks compared with 22½ weeks in expt I—nine weeks after the commencement of tarring 92.4 per

TABLE IV

Experiment II Interval between wart appearance and the development of malignancy

	Number of mice	Interval between appearance of wart and development of malignancy (weeks)	
		Average	Range
Females with mammary carcinoma	5	8.6	2-20
Females without mammary carcinoma	62	8.2	3-22

TABLE V

Experiment II Development of breast cancer

Calendar months		7	8	9	10	11	12	13	14	15	16	17	18
Tarred	Survivors	70	68	56	45	25	14	6	5	5	1	1	1
	No developing breast tumours			1	2	0	1	2	1	0	0	0	0
Control	Survivors	68	67	66	64	63	60	60	57	52	47	42	41
	No developing breast tumours			1	0	2	2	4	7	1	3	0	2
	Percentage incidence of breast tumours in survivors			1.5	0	3.2	3.3	6.7	12.3	1.9	6.4	9.5	12.2

Calendar months		19	20	21	22	23	24	25	26	27	28	29
Tarred	Survivors	0										
	No developing breast tumours	0										
Control	Survivors	36	31	21	16	14	12	5	2	2	1	0
	No developing breast tumours	0	2	3	1	0	0	1	0	0	0	0
	Percentage incidence of breast tumours in survivors	8.3	6.5	14.3	18.8	7.1	8.3	20	0	0	0	0

It is interesting to compare these results with those of Kreyberg who used the same strain of mice, developed from a single female. Of 475 females in the whole strain, 122 developed breast cancer. It has already been pointed out that this incidence is very near to that obtained in this laboratory. When the tar cancer incidence in the breast cancer and non-breast cancer groups was compared, the individuals with breast cancer showed a considerably later appearance and a lower incidence of tar cancer. In this they resembled the males of the strain. The method of calculation eliminated the effect of age. Males of a line in which many of the females developed breast cancer showed an even greater lag in the development of skin cancer. As pointed out by Cramer, females of this line were especially resistant to tar cancer, with the result that only two females bore both types of tumour. In the whole strain, however, at the fifteenth month, 15 out of 26 females with breast cancer had also tar cancer. No explanation of the difference between Kreyberg's results and those of the experiments reported here can be offered. While the larger numbers in his experiment make his results more valuable, it is possible that the segregation into lines so heavily affected by and so completely free from breast cancer accentuated differences in the skin response to tar which would not be seen in mice from that part of the strain where the breast cancers are distributed at random.

Pybus and Miller (1938) reported that, in a line of mice derived from the Simpson strain and especially liable to develop bone sarcomas, multiple spontaneous neoplasms (some benign) were frequent, but that the incidence of mammary cancer was less than in the parent strain. No explanation of this reduction was offered and it is obvious that many factors other than an antagonism between bone sarcoma and mammary cancer may be involved.

The question of the co-existence of two or more independent malignant neoplasms in man is a subject which has received increasing attention in recent years. Up to the time of Murray's Linacre lecture (1927) it was generally agreed that the presence of one malignant tumour exerted an inhibitory effect upon the appearance of subsequent cancers. Murray himself showed that the removal of a tar cancer from one area of the skin of the mouse or of a breast cancer resulted in an indifference of the rest of the skin to tar painting. In 1930, Orr investigated the frequency of occurrence of multiple malignant neoplasms *post mortem* at St Mary's Hospital and brought evidence to show (p. 289) "that there is no aetiological relationship between such tumours, and that they occur purely as the result of coincidence". Warren and Gates (1932) collected and sifted a large series of cases of multiple

malignancy in these warts delayed. Males showed no difference in wart development but their warts took longer to become malignant. The number of animals in this experiment was sufficiently large to warrant such a conclusion.

In expt II, although wart development appeared to be delayed by the presence of a breast cancer, the interval between wart development and malignancy was not increased. The number of mice developing breast cancer was very small.

In expt II there was no evidence that the presence of a tar cancer either increased or diminished the incidence of breast cancer. Although the conditions of the two groups of mice were very carefully controlled, the experiment failed owing to the fact that breast cancer was late in developing in both groups.

These results differ from those of other workers and especially from those of Kreyberg, who worked with mice of the same strain. The difficulties of interpreting the results when small groups of mice are used are discussed.

Some facts about the development of breast cancer in females of the white label strain are given.

It was found that the evidence from the examination of multiple malignant neoplasms in man did not support the view that there is an antagonism between the development of malignancy in two different organs.

We desire to express our gratitude to Dr M. Young for his invaluable and generous help in the statistical analyses of these experiments. We also thank Mr Charles Clarke for his unremitting care of the animals.

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Two membranes with confluent lesions were finely ground with quartz powder and emulsified in 10 c.c. of nutrient broth. The material was centrifuged for 10 minutes in a small centrifuge (3000 *r.p.m.*) and the supernatant removed for stock virus. This was kept in the refrigerator (4° C) and dilutions prepared in saline containing 10 per cent normal horse serum and chilled in an ice bath. Counts obtained from this suspension under various conditions are shown in table I.

TABLE I

Pock counts obtained from a stock suspension of herpes virus.

Date	Dilution	Pock counts from 0.05 c.c.
18.7.38	1:1000 1:10,000	35, 36, 47, 48 2, 4, 4, X
20.7.38	1:2000	6, 8, 10, 12, 12, 13, 14, 16, 18, 18, 20, 21, 21, 23, 27, 29, X, X
21.7.38*	1:2000	(0, 2), 23, 23, 24, 25
21.7.38	1:2000	20, 22

* The same dilution as used on 20.7.38 retested after 24 hours in the refrigerator

X = counts impossible owing to secondary foci

If half values are taken from the results with 1:1000 virus the over-all average for 1:2000 dilution is 18.7. The distribution of counts shows a skew deviation and the median value is 20. In all large series of counts a few very small values are obtained on oedematous or otherwise unsatisfactory membranes, and it is our practice in calculating average values to exclude any counts which are lower than 20 per cent of the mean of other counts with the same material. Only two such counts, bracketed in the table, are excluded from this series. The standard deviation is 5.8, giving a coefficient of variation of 31 per cent. With four eggs to each mixture a difference of 50 per cent. between two experimental results is therefore probably significant, and we have used this as the minimum difference to which significance can be ascribed in the present experiments.

The time required for herpes virus to make effective contact with susceptible chorio-allantoic cells

In interpreting the kinetics of virus neutralisation reactions it is necessary to have some idea of the time which must elapse between placing the virus on the chorio-allantois and the initiation of a lesion. In practice what has to be determined is the time which must elapse before the appearance of a focal lesion fails to be prevented by the addition of a large excess of immune serum. A series of eggs is inoculated with a small (0.02 c.c.) volume of dilute virus, and at intervals larger volumes (0.05 c.c.) of immune serum

being used for each mixture in all the reported experiments. Membranes were removed and foci counted after 44-48 hours' incubation at 35° C.

It soon became apparent that time of contact between virus and serum played a much more significant part in determining the results than was the case with most of the viruses with which we have previously worked. With serum undiluted or diluted 1/2 very little change with time was evident, but a fall was easily demonstrable with higher dilutions. Fig. 1 shows the time

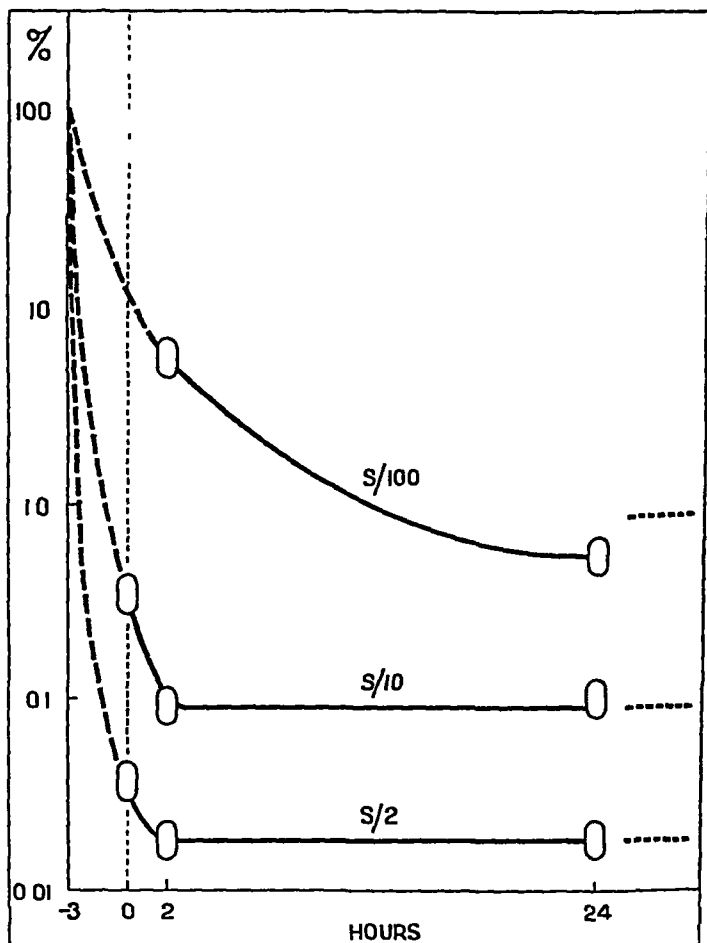


FIG. 1.—Course of inactivation of herpes virus H F by homologous rabbit immune sera

The percentage of foci produced is shown on a logarithmic scale against the time elapsing between making the serum-virus mixtures and inoculating them on to the chorio-allantois.

Ovals represent average pock counts (as percentage of control) at the time shown, the vertical diameter giving approximately the standard deviation of counts. Dotted horizontal lines to the right represent the theoretical equilibrium levels equivalent to the three serum concentrations used. The point from which the curves diverge is arbitrarily placed three hours before the actual time of mixing to allow for the period elapsing after inoculation before foci are initiated.

S/2, S/10 and S/100 = serum diluted 1/2, 1/10 and 1/100

correct an equilibrium mixture of strong reagents should, when diluted 1:10 and allowed time to reach a fresh equilibrium, give pock counts with the same average value as that obtained from the undiluted material. It will be seen from table III that the 2-hour counts of S/2+V and S/20+V/10 are substantially identical, as are also the counts from the immediate test of the 1:10 dilution of S/2+V made at 2 hours (3). The counts from the 1:10 dilution subsequently fall to about half this value. The result when a dilution is made from mixtures which have been 24 hours in contact (4) is quite different. The immediate counts are only about one-tenth of the equilibrium value, but 2 hours later they have reached the same level as the 24-hour counts of the original mixture. Finally there is a sharp fall. We have no explanation

TABLE III

Course of serum-virus reaction as shown by dilution experiments

	Pock counts obtained at the times shown		
	Immediate	2 hours	24 hours
Primary mixtures			
1 S/2+V	11, 15, 20, 20, 22, 27, (0.031)	13, 19, 21, 25, 30, 30, (0.041)	2, 3, 6, 7, 8, 15, (0.012)
2 S/20+V/10	42, 44, 50, 58, 60, 62, (0.088)	15, 20, 21, 26, (0.033)	3, 11, 13, 14, 14, 15, (0.02)
1:10 dilutions of 1			
3 Made at 2 hours	12, 15, 22, 23, 25, 30, (0.035)	4, 5, 6, 7, 14, 27, (0.0175)	4, 5, 8, 11, 14, 16, (0.016)
4 Made at 24 hours	(0), 2, 2, 2, 2, 3, (0.037)	1, 5, 6, 9, 10, 12, (0.012)	0, 0, 0, 1, 1, 2

S/2 and S/20 = serum diluted 1:2 and 1:20

V = virus undiluted

V/10 = virus diluted 1:10

Bracketed figures = percentage of survivors

to offer at present for the rather sharp subsequent falls observed in both sets of dilutions, but the early behaviour would indicate that at 2 hours a condition of equilibrium has been reached which can very rapidly attain a fresh equilibrium on dilution. When primary contact is increased to 24 hours the state of the system has altered in such a way that a new equilibrium takes a good deal longer to be reached after dilution. This may be expressed in the familiar form that antigen-antibody union becomes firmer with the lapse of time, but it is not easy to interpret in terms of the reversible system such as has been established at least of the virus-antibody reaction in general where there is practically no change in the results between 2 and 24 hours, dilutions made

was obtained by corneal inoculation in the rabbit and adapted to egg passage without much difficulty. The titre of this virus

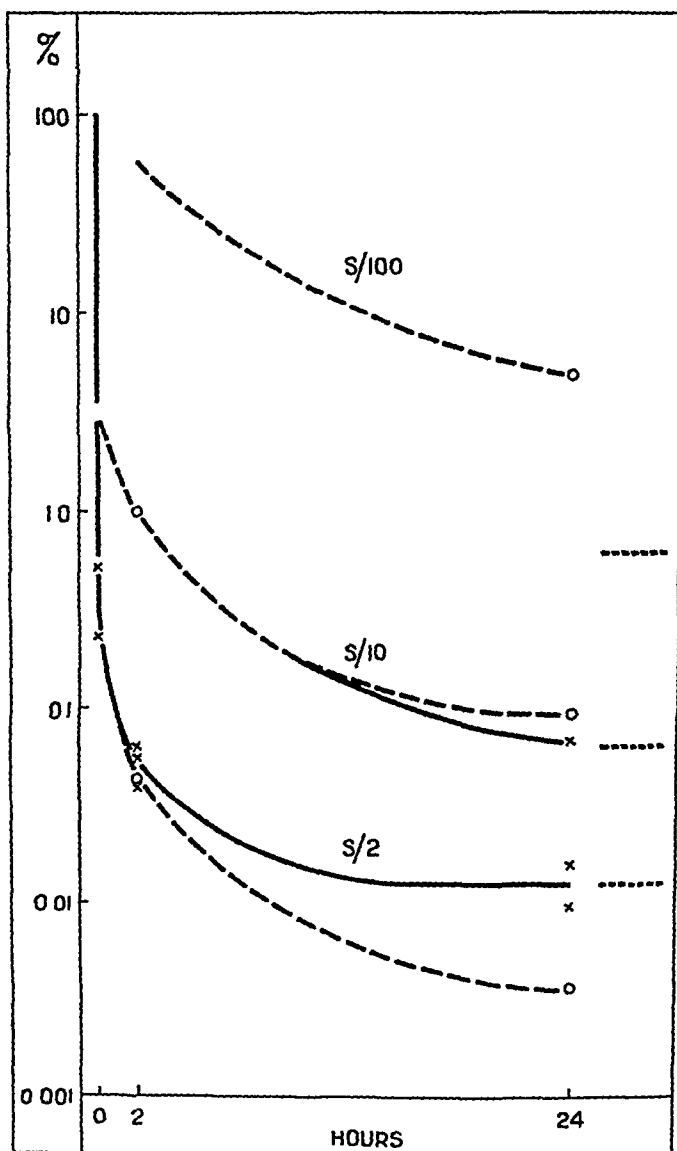


FIG. 2.—Course of inactivation of virus H F by human immune serum "Tr"

Data from two experiments (solid and broken lines) are combined, points shown being percentage values deduced from the average of pock counts. General arrangement as in fig. 1

was, however, considerably lower (one-tenth to one-hundredth) than that of H F egg virus. The serum was tested against both H F and the homologous virus Tr. Fig. 2 combines the data

Discussion.

For reasons which have been discussed elsewhere (Burnet, 1936) the chorio-allantois of the developing egg is the most suitable tissue for the titration of those viruses which are capable of producing satisfactory lesions thereon, and its use has allowed a closer study of the details of virus-serum reactions than would be practicable by any other current method. The HF strain of herpes, with which most of the present work has been carried out, has been the most satisfactory virus to titrate on the chorio-allantois that we have yet encountered. The lesions are easily visible but not too large and are rarely obscured by the development of secondary foci.

As a preliminary to further work on the nature of immunity to herpes virus in man and experimental animals it was necessary to determine the most suitable technique for titrating the virus-inactivating antibody of immune sera. At the same time an attempt has been made to determine whether the antibody-virus reaction conforms to the type found with similar reactions involving other viruses. A recent monograph from this laboratory (Burnet, Keogh and Lush) has been devoted to an interpretation of these reactions, the data used being derived in part from the large amount of work by others in which orthodox animal inoculation methods have been used, but predominantly from our own studies with the chorio-allantoic technique. The conclusions reached were in general accord with those of earlier workers in the field, the greater accuracy and convenience of the chorio-allantoic technique merely allowing a more detailed elucidation of relatively minor points. Virus inactivation by immune serum results primarily from union of antibody to the virus surface. This union is, however, a fully reversible one which progresses and reaches an equilibrium level determined by ordinary chemical considerations. The observed inactivation cannot therefore be a simple killing of virus by antibody but is the result of an interaction between the susceptible cells used for titration and the antibody-coated virus particles. The only controversy likely to arise in regard to such a general statement is as to whether a real virus-antibody union occurs *in vitro* or whether the whole inactivation must be regarded as a threefold interaction of virus, antibody and susceptible cell, no union between virus and antibody occurring *in vitro* (cf. Sabin, 1935). In the monograph referred to, evidence that virus-antibody mixtures showed different degrees of infectivity according to the time they had been in contact was brought forward as proving that union *in vitro* did occur.

Attempts to demonstrate such influence of time of contact are complicated by the fact that after inoculation of mixtures in or on

4 After inoculation of virus on to the chorio-allantois a period of 2-4 hours must elapse before an excess of immune serum will fail to prevent the initiation of a specific focus

5 The inactivation of herpes virus by immune rabbit and human sera has been studied quantitatively by the chorio-allantoic technique

- (a) When sufficient time is allowed, an equilibrium level is reached at which the product concentration of immune serum \times percentage of survivors is constant
- (b) The time necessary to reach this level is inversely related to the concentration of immune serum
- (c) For some time after the equilibrium level is reached, dilution experiments show that the union is freely reversible
- (d) With strong virus-serum mixtures a subsequent development of partial irreversibility may occur.

6 The inactivation of herpes virus by natural human immune serum progresses at a slower rate than with rabbit immune sera of the same final titre

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then neutralised with dilute HCl. The effect of this treatment is to liberate the kallikrein from the inactivator to which it is bound in the serum.

Five samples of serum from healthy adult individuals were employed and 10 c.c. of each were treated as described. The solution obtained was again precipitated with six volumes of acetone, washed with acetone and ether and dried at room temperature. The next day the five samples of powder were each extracted with 5 c.c. of 0.9 per cent NaCl and dilutions of 1:2 and in two cases of 1:10 were made. These solutions and a control solution of 0.9 per cent NaCl were tested for spreading activity in rabbits.

Each solution was mixed with an equal amount of an indicator, either trypan blue (1 per cent) or diphtheria toxin (1:1000) in 0.9 per cent NaCl, and 0.25 c.c. of each mixture was injected intradermally, a single row of three or four injections being placed on either side of the vertebral column. The backs of the rabbits had been shaved 24 hours previously and showed no signs of irritation at the time of the test. The areas of blue discolouration or of inflammation were measured 48 hours later by cutting out pieces of paper of exactly the same size and measuring these with a planimeter, the results being expressed as sq. cm.

Results.

It is seen in the table that a moderate spreading activity is exhibited by extracts obtained from serum treated in the manner described.

In view of the statement of Duran-Reynals (1936) that some commercial peptones possess moderate spreading activity, it is possible that such unspecific factors might be responsible for the results obtained. On the other hand the same author points out that it is possible that peptones owe their activity to contamination with factors from tissue. This suggestion is supported by the fact that padutin, which is made from urine and has its origin in the blood, is a spreading factor ranking with acetone-precipitated and dialysed testis factor.

If kallikrein is the only spreading factor in serum, one would not expect a greater effect than was obtained in these experiments, for its concentration in serum is relatively low. Serum contains 1-2 kallikrein units (K.E.) per c.c. and only by complicated methods can it all be liberated. Accordingly the highest possible content of active kallikrein in 0.25 c.c. of the mixture of undiluted extract with indicator would be 0.50 K.E. By titrations to the end-point, I have determined that padutin solutions of similar K.E. content exhibit a similar or somewhat more marked spreading activity. It must be borne in mind that the serum preparations used in these experiments were very impure, so that adsorption might have played a role in diminishing the spreading.

Titration of the spreading activity of padutin with diphtheria toxin as indicator showed a distinct effect in dilutions of 1:1000 of an original standard solution containing 30 mg. per c.c. In

although the final areas obtained with solutions of the same concentration of testis extract, purified by acetone precipitation and dialysis, and of padutin are of equal size. No difference between the two factors could be discovered in titrations to the end-point

Another circumstance to be mentioned is the strong œdema-producing activity of padutin solutions of sufficient concentration (10 mg or more per c.c). In this respect also padutin resembles purified testis extract.

Kallikrein can be prepared in a somewhat purer form than that present in padutin, but such preparations are very unstable. It has not been possible to obtain such a preparation or one from pancreas and it is hoped that workers dealing with spreading factors if they have the opportunity to examine highly purified kallikrein will investigate its spreading activity.

Summary

1 When serum is treated by a method suitable for the recovery of kallikrein the product obtained produces a spreading effect on the diffusion of trypan blue and diphtheria toxin in the skin of the rabbit

2 The effect is quantitatively comparable to what is produced by kallikrein in the concentration present in serum.

3 Kallikrein does not appear to be identical with the spreading factor obtained from the testis

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to rabbit cells. The β staphylolysin according to Bigger, Boland and O'Meara (1927), Glenny and Stevens (1935), and Bryce and Rountree (1936) is characterised by the hot-cold lysis of sheep cells and has no antigenic relationship to the α toxin. The α_1 and α_2 toxins of Morgan and Graydon (1936) have not been sufficiently studied. Therefore the discussion in this paper centres around the classical α staphylohaemolysin and its relation to the lethal and dermonecrotic manifestations of this staphylotoxin.

Neutralisation of toxic effects by standard serum

The first series of experiments was carried out with toxin A, which was prepared about 3 years ago from strain C 24. In dry form its assay gave the following results.

Lh, the amount of toxin which when added to 1 unit of U.S. standard antitoxin produces 50 per cent haemolysis of 1 c.c. of 1 per cent rabbit cells, = 0.001923 g. of the dry toxin.

HU, the amount which alone produces 50 per cent haemolysis of 1 c.c. of 1 per cent of rabbit cells, = (average of 100 determinations) 0.0000025 g.

Lr, the amount which when added to 1 unit of standard antitoxin will produce discernible skin necrosis in 50 per cent of guinea-pigs injected intradermally, = (average of numerous tests) 0.002375 g.

RU, the amount which alone will produce visible dermonecrosis in 50 per cent of guinea-pigs injected intradermally, = (average of numerous tests) 0.00045 g.

L₊, the amount which when added to 1 unit of U.S. standard antitoxin will cause the death of approximately 50 per cent of white mice injected intraperitoneally, = (average of numerous tests) 0.002575 g.

U₊, the amount which alone will cause death in 50 per cent of white mice injected intraperitoneally, = (average of numerous tests) 0.000655 g.

By subtracting the values of HU, RU and U₊ respectively from the values of Lh, Lr and L₊ the same value of 0.00192 g. is obtained. This indicates that 1 unit of U.S. standard staphylococcus antitoxin exactly neutralises the haemolytic, dermonecrotic and lethal properties of 0.00192 g. of dry staphylotoxin. It is therefore tentatively concluded that lysis of the red blood cells of the rabbit, necrosis of the skin of the guinea-pig and acute death of white mice are caused by the same toxic constituent of the staphylococcal filtrate.

Titrations of various concentrated commercial antitoxins

If this conclusion is correct, the titration values obtained for a large number of concentrated antitoxins prepared from a great variety of toxoids should be the same for the lethal and dermonecrotic titrations as for the haemolytic. Experimentation suggests that the discrepancies reported by many previous authors were

In preliminary experiments it was found that the presence of hæmoglobin did not interfere with absorption nor with the quantitative estimation of the various activities of the toxin after treatment with red blood cells. In the following experiments intact rabbit red blood cells were used as the absorbing agent.

Dilutions of toxin A were prepared in such a way that 0.2 c.c., as used in the dermonecrotic series, or 0.5 c.c., as used in the lethal series, contained the doses of toxin indicated in column 2 (toxin dose) in table II. These were subjected to no treatment and were placed in the cold room as controls. Another set of dilutions was prepared in such a way that 0.1 c.c. in the dermonecrotic series and 0.25 c.c. in the lethal series contained the same doses of toxin. To these dilutions were added equal volumes of highly concentrated suspensions of rabbit red blood cells. For technical reasons it was found necessary to use different concentrations of cells for each series. The treated toxin dilutions were then placed in the incubator for one hour and in the cold room overnight. The undissolved cells were found to have settled down well to the bottom of the tubes. For further separation of the supernatant fluid, the tubes were centrifuged and the clear fluid drawn off by means of fine pipettes. From each toxin dilution of the dermonecrotic series 0.2 c.c. was injected intradermally into each of 10 guinea-pigs and from each dilution of the lethal series 0.5 c.c. was injected intraperitoneally into each of 10 mice. The results are recorded in table II.

TABLE II

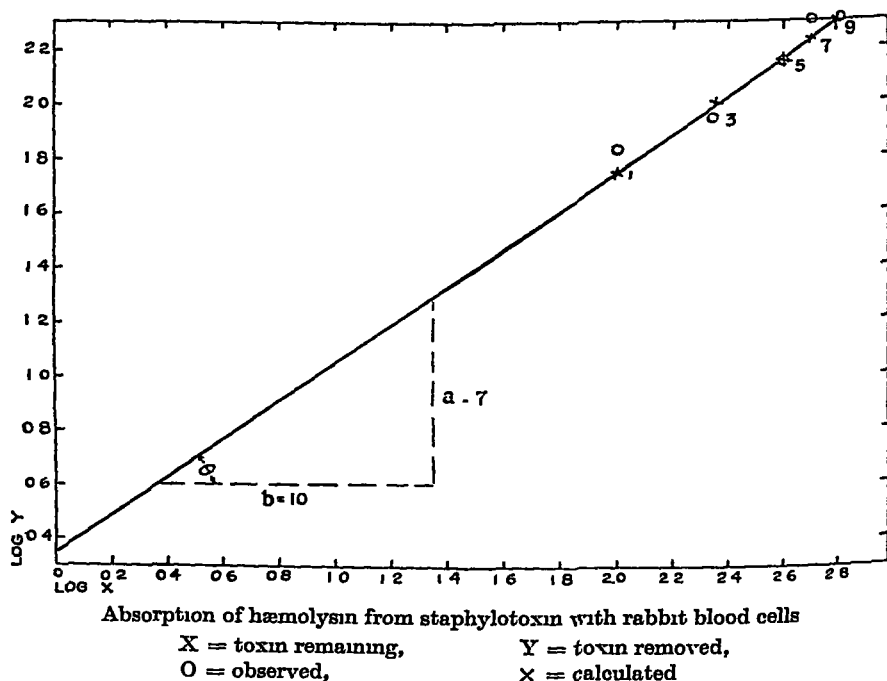
Effect of absorption with rabbit cells on the dermonecrotic and lethal action of staphylotoxin

Toxin dilution no *	Toxin dose (g.)	No. of positive dermonecrotic reactions		No. of mice killed by injection of toxin	
		Before treatment	After treatment	Before treatment	After treatment
1r-1+	0.00020	0	0	0	0
2r-2+	0.00025	0	0	0	0
3r-3+	0.00030	0	0	1	0
4r-4+	0.00035	1	0	1	0
5r-5+	0.00040	3	1	2	0
6r-6+	0.00045	5	1	2	1
7r-7+	0.00050	9	3	3	0
8r-8+	0.00055	10	6	3	0
9r-9+	0.00060	10	10	4	1
10r-10+	0.00065	10	10	5	1
11r-11+	0.00070	10	10	10	3
12r-12+	0.00080			9	5
13r-13+	0.00090			10	10
14r-14+	0.00100			9	10
15					
15r-15+	0.00125			10	10

* 1r, 2r etc. refer to dilutions of the dermonecrotic series, 1+, 2+ etc. to those of the lethal series.

It is seen from this table that, before treatment, 0.2 c.c. of toxin dilution 6r, containing 0.00045 g. of dry toxin, represented the Lr dose, and 0.5 c.c. of dilution 10+, containing 0.00065 g. of dry toxin, represented the L+ dose. This is in accordance with

50 per cent of the mice injected. From the lethal series of table II it is seen that prior to treatment this end-point was given by solution 9+. Referring to table III, it is found by calculation that one dose, or 0.5 c.c., contained 260 HU. Again, from table II it is seen that, after treatment, the end-point was given by solution 12+. Referring to table III, it is found by calculation that each dose (0.5 c.c.) contained 350 HU before treatment and 250 HU after. The same was found to be true when similar series of titrations were carried out with the sets used in the dermonecrotic experiments. Analysis of other data presented in tables II and III showed not only that there was a reduction in the dermonecrotic and lethal intensities of the staphylo toxin following its treatment with rabbit red blood cells, but that this reduction is proportional to the reduction in hæmolytic activity. All follow the same quantitative law which is derived by plotting the logarithmic values (fig.) of



X and Y (observed) recorded in table III. A sloping straight line is obtained which can be interpreted as described elsewhere (Levine, 1938b). It will then be found that the quantitative relationships of the toxin consumed to the toxin remaining in solution can be expressed by the following mathematical generalisation

$$\begin{aligned}\log Y &= \log 2.2 + 0.7 \log X, \text{ or} \\ Y &= 2.2 X^{0.7},\end{aligned}$$

where Y = the number of hæmolytic units of toxin consumed in the process of treatment, X = the number of hæmolytic units

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hard and not tender, and it extended below the umbilicus, the liver extended 2 ins below the right costal margin and felt soft. There were no enlarged lymph glands. The heart was slightly enlarged to the left. Both lungs showed signs suggesting basal compression. Nothing unusual was found in the central nervous system or urine. Blood count—red cells 3,030,000 per c mm, hæmoglobin 45 per cent, colour index 0.75, white cells 34,500 per c mm (polymorphonuclears 43.5, lymphocytes 12.5, monocytes 4, eosinophils 1, metamyelocytes 9, myelocytes 28, myeloblasts 1.5 per cent), reticulocytes 4.8 per cent, platelets 149,000 per c mm. The nucleated red cells were 79,000 per c mm and the varieties present were normoblasts A (primitive) 0.5, normoblasts B (polychromatic) 15, normoblasts C (orthochromatic) 55, megaloblasts B (Ehrlich type) 25, megaloblasts C (pyknotic) 4.5 per cent. The Price-Jones curve showed a mean red cell diameter of 6.16 μ , coefficient of variability 11 per cent, microcytosis 38 per cent, megalocytosis nil.

Sternal marrow biopsy was performed by the puncture method and the smears showed the following percentages of cells:

Polymorphonuclears	15.4	Normoblasts	52.4
Eosinophils	Nil	Type A	3.2
Basophils	0.4	„ B	12.0
Metamyelocytes	3.4	„ C	37.2
Myelocytes	7.4		
Myeloblasts	1.4	Megaloblasts	14.2
Monocytes	1.8	Type B	9.0
Lymphocytes	3.4	„ C	5.2
Plasma cells	0.2		

Illustrations of the cells in the blood and marrow are given in figs 1-4.

Fractional gastric analysis showed hypochlorhydria, the Wassermann reaction was negative, there were 2 units of bilirubin in the serum.

Radiographic examination of the skull and long bones revealed no abnormalities apart from some porosity of the tibiae and fibulae, there was no evidence of myelomatosis.

The clinical findings and the blood picture had suggested that the patient might be suffering from a chronic myeloid leukaemia with unusually large numbers of nucleated red cells in the blood. But the sternal marrow result decisively negated this suggestion, since in leukaemia the characteristic change in the marrow—whatever the peripheral blood may show—is a great preponderance of leucocytes, whereas this patient had twice as many red cells as white cell types. The diagnosis seemed to lie between a disturbance of red-cell-forming tissue allied to the leukaemias or a leuco-erythroblastic anaemia.

Various forms of therapy were attempted, as shown in the last column of the table, but none seemed to influence clearly the course of the disease, even a blood transfusion had little effect. The anaemia pursued a fluctuating course for 4 months, the numbers of white and nucleated red cells also fluctuated, but the latter were always much more numerous than the former. The types of nucleated red cells present did not vary, nor did their relative proportions vary very greatly.

On 28th February 1938 the patient was sent to the Infirmary's convalescent hospital where she remained for a month, and then, since she had been 4 months in hospital, she was allowed to go home. After 31st March she failed to attend the department's clinic and her death was reported by her doctor shortly afterwards. Permission for an autopsy could not be obtained.

Discussion.

The pathological condition affecting this patient was unusual and the possibilities worth consideration are as follows.

1. *Leuco-erythroblastic anæmia* This type of anæmia occurs most commonly as a result of malignancy or sclerosis in the bone-marrow. The characteristic features have been described by several writers and according to Vaughan are . anæmia not severe, leucocytosis not marked, immature red cells including Ehrlich's megaloblasts constantly present together with a few immature leucocytes. The patient described here showed quite a different picture apart from the presence of Ehrlich's megaloblasts. Allen and Childs (1936) have described a rather similar case, but much less severe and having only a mild anæmia; they suggested that it might be an adult variety of Cooley's leuco-erythroblastic anæmia although they admitted that the characteristic radiographic changes in the bones were absent.

2 *Erythroblastic disease of adults* In this syndrome, described by Émile-Weil and Perlès (1938), the patient has enlargement of the spleen and liver and immature red cells appear in the peripheral blood. The spleen and liver contain large numbers of erythroblasts (normoblasts). The sternal marrow, on the other hand, is hypoplastic. This alone excludes the patient under discussion from this group, and there are other differences, *e.g.* the patients with erythroblastic disease were not so severely anæmic and the normoblasts in the blood were never more than 12 per cent of the leucocytes.

3 *Polycythæmia vera* This disease has been known to end in an anæmic stage, but it is a surprising fact, as shown by Vogel, Erf, and Rosenthal (1937), that proliferation of normoblasts in the marrow is not found, and megaloblasts have so far not been seen at all.

4. *A leukæmia-like condition* It is possible that the pathological condition in this patient was a proliferative state of the immature red cells standing in the same relation to the red cell series as leukæmias do to the white cell series. Di Guglielmo (1928, 1935, 1938) has described under the name of "acute erythræmia" a group of patients clinically resembling acute leukæmia but, instead of primitive leucocytes, large numbers of immature red cells appear in the blood and infiltrate the spleen, liver and other organs. As many as 143,000 immature red cells per c mm have been recorded in adult patients, there is a leucocytosis—up to 30,000 per c.mm—but the differential count remains normal. The cells are of the normoblast series and there are also very primitive erythroblasts. Di Guglielmo's (1928) illustrations do not show any megaloblasts, but Penati (1937) has recorded a case in which many megaloblasts

also isolated by Hall and considered to be a species of *Glaucoma* but different from the foregoing, and (4) C S, isolated by Elliott (1933) and considered to be *Colpidium striatum*

The determination of the species of these ciliates is so difficult that the writer proposes to make no attempt to do so along taxonomic lines. It will suffice for the moment that the strains all belong to the *Glaucoma-Colpidium* group and the reader is referred to the work of Chatton (1939) for further details. The serological evidence to be adduced in the sequel goes to show that the strains Gl R and C S are so exactly similar in their reactions as to be considered examples of the same "species" and certainly as belonging to the same serological variety. The three other strains used are serologically distinct from each other and from Gl R and C S.

The method of culture is extremely simple and the organisms grow very well in Erlenmeyer flasks of any size from 50 c c upwards. The best medium proved to be Difco proteose peptone 1 per cent in glass-distilled water with the addition of 0.6 per cent NaCl.

Preparation of the sera

This presented no difficulties. The living organisms were centrifuged down once, great care being taken not to let them be packed into masses at the bottom of the centrifuge tube. They were resuspended in 0.5 per cent NaCl and immediately injected into the ear vein of the rabbit. Between 15 and (about) 33 million living ciliates were injected in 4 or 5 doses and the animal was bled 5 days after the last dose. The glaucomas do not tolerate the action of the centrifuge very well and care had to be taken to damage them as little as possible before they were injected.

Technique of the test used.

It is important in work of this kind to find out what is the most appropriate form of test to use. Titrations could be carried out very well in small tubes containing descending concentrations of serum diluted with 0.5 per cent NaCl to which glaucoma suspensions were then added. The tubes were left at room temperature in the dark or well shaded from direct daylight and were read with a hand lens at intervals from 1 to 5 hours and again at about 22 hours. This method gave satisfactory tests with living organisms, but the macroscopic observation suppressed a great deal of valuable information.

Parallel tests were therefore always set up by placing 1.5 c c of the serum dilutions in descending series in ordinary test tubes and after 1.5 c c of the glaucoma suspension had been added, the tubes were immediately tipped out into numbered petri dishes $1\frac{1}{2}$ in in diameter. These were placed in a glass container at room temperature shielded from the light and were read under the low powers of the microscope ("aa" and "A" Zeiss objectives) at suitable intervals. In these experiments it was essential to centrifuge the sera, which should be sterile, immediately before making the dilutions.

Preparation of the test suspensions

The preparation of the test suspensions proved to be of the greatest importance. Owing to the relatively large and very variable size of the organisms it was found essential to have perfectly healthy and actively swimming organisms, which would remain in this state in all negative readings and in the saline controls without any deaths for at least 24 hours. Glaucomas can be fixed by a method to be described later so as to preserve their shape

were here more clearly appreciated and it will be best to describe (1) a slight to moderate reaction with a not very potent serum, for example Gl R with its homologous serum, and (2) a severe reaction such as that seen in experiments with St L and the homologous serum no 71

It should be remarked in general that agglutination is always best seen in tubes and may be less well marked in the plates containing the highest concentration of serum than in those with a somewhat lower concentration. One must remember however that the sedimentation of immobilised or otherwise affected organisms was interpreted, not always justifiably, as agglutination and added to the apparent titre in the tubes

1 *Tests with strain Gl R.*

In the plates the actively swimming glaucomas (Gl R) which had been in contact with the serum for 5-10 minutes showed in the concentrations 1 10-1 160 a rapid loss of motility and a characteristic very slight shrinking of the outer pellicle. They gradually agglutinated or lay singly on the bottom of the glass. An almost universal reaction was the early emission of a transparent exudate containing minute brightly refractile particles. With less potent sera the faintly motile organisms came together in oscillating clumps held together lightly by the amorphous exudate, while the still slightly motile cilia showed beads of this substance upon their surface. Rather stout strands of somewhat more refractile material were frequently seen at the posterior ends and these appeared to anchor the ciliates to each other.

If the effect was more severe the immobilised glaucomas showed a thickening of the pellicle, either in the form of a uniform sheath or appearing as fragments of the same substance as the sheath, thus giving the organism a rough sticky appearance. In the plates containing lower concentrations of serum the agglutination was well developed, with exudate and beads of material on the cilia, and the glaucomas were rarely completely immobile. After 5-6 hours there were usually some dead individuals in the clumps. In the higher concentrations, the number varying with the potency of the serum, but there now also occurred a progressive recovery of the surviving organisms. In spite of the sticky rough excrescences on the pellicle the cilia began to beat with sufficient force to produce some degree of swimming and the agglutination gradually dispersed, leaving the dead clumps and the masses of free but agglutinated exudate on the bottom.

The organisms are normally provided with a pellicle from which the cilia emerge as slender thread-like processes. The serum clearly had a direct effect upon the alveolar layer immediately below the pellicle, in which lie the trichocysts, and possibly, though

Lwoff's (1936) silver impregnation method and counterstained with toluidine blue or thionin. These showed all the exudate and sheath material stained pink in contrast to the blue staining of the protoplasm, suggesting that it might be of the nature of mucin, a supposition agreeing with Bresslau's results to be discussed later. They showed further that with heated serum there was no direct attack on the cilia. The exudate had emerged and was visible as pink-staining globules and it seemed that the sheaths were continuous envelopes possibly of the same substance. The complicated subciliary structures which are so well revealed by the silver method and which comprise the "cinetome" (*i.e.* the basal granules of the cilia and their connecting strands) and the

TABLE I

Titration of heated immune sera with homologous organisms

Serum	1 20	1 40 1 80	1 160	1 320	1 640	1 1280	1 2560	Control (saline)	Time (hours)	Test suspension.
65 (living Gl R) test 116	++++	++++	++++	+++ (+)	+++	+	(+)	—	25	Living Gl R, 3000 per c
	++	+	(+)	(—)	—	—	—	—	22	
68 (living Gl R) test 275	++++	++++	++++	+++ (+)	+++	++	+	—	25	Living Gl R, 2500 per c
	+++	++	++	+	tr	tr	—	—	22	
71 (living St L) test 152	++++	++++	+++ (+)	+++ (+)	+++	++	++ (+)	—	25	Living St L, 2500 per c
	++++	++++	+++	+++	++ (+)	++ (+)	+	—	22	

The readings are from the tube series and give the macroscopic findings

"argyrome" (*i.e.* the silver line system), were not cast off with the secreted sheath. That the structures of the argyrome play some part in the secretion of the exudate and of the sheath appeared to be probable, but this is an intricate matter requiring separate study.

Summary of the effects of heated immune serum

The direct effects of the contact of heated immune sera with the homologous organisms can be summarised as due to a combination of the serum with the surface structures so as to cause (1) a greater or less degree of immobilisation, (2) the production of a sheath or envelope which may be cast off, (3) the pouring forth of an exudate, apparently from the alveolar layer below the cuticle, which passes out into the surrounding medium and combines with the serum, (4) a temporary or permanent agglutination of the

with GI R, while the titre of fresh rabbit sera was about 1 : 40 ; with St L. the titres were rather higher

As the concentration of the serum diminished, the effect usually terminated rather abruptly and the disappearing reaction was registered as the death and lysis of a small proportion of the individuals while the rest were unaffected, so that in those sera where the concentration was too low for lysis to take place, there was no other response such as the formation or discarding of sheaths, temporary agglutination or the exhibition of beaded cilia

TABLE II
Effect of absorption with Glaucoma at 0° C on action of normal guinea-pig serum

Action upon suspension of <i>Glaucoma</i>							
Serum	1 10	1 20	1 40	1 80	1 160	Saline control	Test suspension and expt no
Absorbed at 0° C with GI R	+++ (+) (nearly all dead and lysed)	+++ (many dead and lysed)	++ (about $\frac{1}{2}$ dead and lysed)	—	—	—	GI R (264)
Not absorbed	++++ (all dead and lysed)	++++	++++	++ (about $\frac{1}{2}$ dead and lysed)	tr	—	GI R (266)
Action as complement in hæmolytic system							
Serum	1 5	1 10	1 20	1 40	1 80	Controls	
Absorbed as above	++++ (complete)	++ (+)	+	tr	—	Saline, I B, and complement controls, no hæmolysis	
Not absorbed	++++ (complete)	+++ (+)	++ (+)	++	+	, " " "	

Read at 5 hours from plates

To get further information concerning the action of normal sera with intact complement, GI R, killed by exposure to 70-75° C. for 45 seconds, was placed in contact with pooled guinea-pig serum overnight at 0° C. The organisms were centrifuged off and the serum was used in tests of the usual kind with living glaucomas, and as complement in a hæmolytic test with sheep corpuscles and the appropriate immune serum. The absorbed serum was found, in comparison with the unabsorbed, to have lost somewhat in titre both as a lytic agent for glaucoma and as hæmolytic complement, but the nature of its action remained unaltered (table II)

It is not claimed that this experiment is entirely conclusive.

of thick sheaths and agglutination and death of all the organisms up to dilution 1 80, but no lysis. Dead individuals were still present in certain cases, though in small numbers, in dilutions up to 1 3000 and agglutination titres were higher than in the heated sera. In lower concentrations faint traces of an attack upon the cell substance could be seen but it did not amount to a frank lysis.

In fresh immune sera made against St L., death without lysis of all the organisms occurred up to dilution 1 640 and many dead were still to be observed in 1 2560. The glaucomas often showed a tendency to burst half out of the sheath and pellicle and died in the half extruded state. The immune sera with intact complement showed enhanced titres of agglutination and were much more lethal than the heated sera, but lysis was not a feature of the reaction and only traces of this were produced in the lower concentrations. These findings suggested that the high immune body content of the sera masked or inhibited the lysis which was so striking a feature of the action of fresh normal sera. This point arises again in later sections of the paper. Lysis in these experiments was taken to mean not merely the bursting out of the pellicle or sheath but an actual attack on the substance of the ciliate, producing dissolution of the protoplasm and peripheral structures.

Effect of the addition of complement to heated sera

In experiments with heated immune sera to which fresh guinea-pig complement is added it is very important to put up the tests in duplicate or triplicate with different concentrations of complement and to be quite certain that the complement controls without immune serum are in perfect condition at the 24-hour reading. It is also necessary to carry the dilution of the amboceptor or immune body far enough, as important changes in the effect appear in the higher dilutions. The number of organisms in the suspensions should be adjusted to not more than 1500-2000 per c.c. in the completed tubes and plates, as the lower number gives a more clear-cut result.

Owing to the difficulty of reading the tubes where lysis has been going on actively, plates were found to give more reliable results. The immune serum was first diluted and distributed in tubes, complement was then added and the racks were placed in the incubator at 37° C. for 20 minutes and then allowed to stand at room temperature for about half an hour before the ciliates were added.

Table IV gives the result of a typical experiment. The points to be noted are that the serum was capable of killing large numbers of glaucomas in the initial concentrations of 1 400 and 1 800 without the addition of complement. The dead organisms showed no lysis in the serum alone. The peak of the effect, which included death as well as agglutination and sheath formation, was seen in the highest serum concentration and diminished with dilution. At dilution 1 12,800 agglutination did not occur and only the

concentration of complement also by itself innocuous to the ciliates. This phenomenon was at first interpreted as an example of combination in optimal proportions later it was found that there was a greater complexity both of antigen and antibody than was at first supposed. Actually a competition for the complement was taking place between these elements and constituted an obscuring factor. This is analysed in detail in the succeeding paper

In tests with added complement where the immune serum was not in itself lethal, as occurred very frequently in sera made with Gl R, there was no first lethal peak in the higher concentrations of the immune serum. The addition of complement produced a certain amount of death without lysis. The effect increased with *dilution* of the immune body up to a given point and the culmination, with maximum death and lysis, always occurred at a dilution where the action of the serum alone was barely discernible.

It must be observed in this connection that the sensitiveness of the test in regard to the actual end-point at which there was no discernible effect in experiments in antisera without complement was only relative, and it cannot be asserted that there was no serum effect at all merely because obvious agglutination or immobilisation could not be recorded.

Summary of the effects of heat-labile factors.

Glaucoma is very sensitive to the heat-labile element in normal guinea-pig and rabbit serum and is lysed when exposed to dilutions up to 1/80-1/160 or more. Fresh mouse serum showed a normal agglutinin which proved to be heat labile. The heated normal serum of these species was without any detectable effect. The addition of suitably diluted complement to immune sera greatly intensified their action, but the effective proportions for the production of lysis of the organisms were found at dilutions of immune body so high that its action was almost inappreciable in the control tests without complement.

THE EFFECT OF RE-EXPOSURE TO HEATED IMMUNE SERUM

In this work the temporary nature of the agglutination in many of the tests and the subsequent growth of the survivors in the tubes where death was not a feature of the reaction led to the investigation of the behaviour of organisms which had been exposed to serum. It was found that, with the addition of peptone water, both Gl R and St L could be cultivated indefinitely in immune serum.

If Gl R was grown in the immune serum peptone water in a concentration of say 1/50 of a not very potent serum and sub-

ABSORPTION OF AGGLUTININS

The agglutinins were successfully absorbed from immune sera made against Gl.R but there were certain technical precautions which had to be taken owing to the nature of the organism

The absorption was made by exposing living organisms which had been spun down once in the centrifuge and re-suspended in saline to immune serum in the incubator at 24° C or at room temperature in the dark. Agglutination took place rapidly and after about an hour the supernatant serum was drawn off and the process repeated 6 times. The serum, now diluted 1/25, was thoroughly centrifuged before use. A total of 43 million organisms was used where complete absorption was obtained. It is very important to centrifuge the suspension very gently so as to destroy as few as possible. It is also essential to remove the serum from the agglutinated glaucomas so that in the early stages there should be a minimal release of protoplasm from dead or injured organisms. In the later stages the pipetting off of the supernatant serum at the height of the agglutination prevents the secondary release of the surviving organisms. In the final stages the ciliates have to be centrifuged off as they are no longer all agglutinating and the process should be carried out with due care.

TABLE V
Absorption of agglutinins

Serum	1 50-1 200	1 400	1 800	1 1600	1 3200	1 6400	Saline	Time (hours)
51 absorbed with Gl R	—	—	—	—	—	—	—	2.5
Test 76	—	—	—	—	—	—	—	22
51 control, treated with saline	++++	+++(+)	+++	+++	(+)	—	—	2.5
Test 77	tr	tr	tr	tr	—	—	—	22
51 absorbed with <i>Polytoma uvella</i>	++++	++++	++(+)	+(+)	tr	—	—	2.5
Test 78	+	tr	tr	tr	—	—	—	22
51 untreated	++++	++++	+++	+++	+	—	—	2.5
Test 79	+	tr	tr	tr	—	—	—	22

The readings are from the tube series and give the macroscopic findings. Test suspension Gl R. 6000 per c c

In the tests summarised in table V the controls consisted of immune serum treated with saline in parallel with the absorption and the same serum diluted directly at the time when the test was put up. A further control was made by exposing the immune serum to very thick saline suspensions of a bacterium-free culture of an unrelated protozoon, *Polytoma uvella*, the process being carried out exactly as in the absorption with *Glaucoma* except that the *Polytoma* did not agglutinate and had to be centrifuged off at every exposure.

inadvisable as, although an exudate similar, so far as it has been tested, to that found by Bresslau has been observed in these ciliates, it is not very helpful to give it a meaningless name. If other protozoa secrete, or have in their surface structure an antigenic substance with similar properties to the exudate of ciliates, as seems not improbable, these will no doubt be specific substances, very probably carbohydrates combined with protein material as in the bacteria, and the general description of mucin can be used to cover them until they can be more accurately described.

In the present experiments the combination of the serum with the exudate and those surface structures concerned with the formation of the sheath was demonstrated beyond any doubt, and it was also clear that those organisms which survived reached a state in which their substance was no longer combining in the same way. This was demonstrated in the re-exposure experiments and the writer considers that the explanation of the insensitiveness to serum shown by the organisms growing in immune serum or surviving in a titration experiment may be due to one of two possible conditions. Either the ciliates had shed all the antigenic substance and were therefore not sensitive until more was produced, which would agree with Bresslau's conception, or the surface of the organism had undergone a temporary change of physical condition which did not admit of the carrying out of agglutination, while still retaining a chemical composition which is not antigenically different. The balance of evidence appears to be in favour of the second hypothesis, for though only a relatively small proportion of the volume of protozoa seems to be antigenic (Kligler and Olitzki, 1936) the complete extrusion of the substance does not seem probable. It is further quite clear that the state of the surface of the organism is of primary importance in all the phenomena of agglutination and of reaction to immune serum in general, and that the surface of an organism capable of such active response should be temporarily altered need give no cause for surprise.

The active lytic effect of normal serum of rabbits and guinea-pigs with complement intact is a particularly interesting phenomenon in view of the absence of any recognisable similar action of normal heated serum. The suggestion that the heat-labile elements of normal sera are capable alone of causing complete lysis without amboceptor or immune body is so contradictory of established knowledge that it calls for further elucidation. It is true that it has been established that red cells may be lysed by fresh guinea-pig serum without immune body in the absence of salt, where the sodium chloride was replaced by sugar (Topley, 1914-15), but the conditions in the experiments here described are not actually similar.

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Methods and material

The method used to obtain antisera was the injection of the antigen however prepared into the ear vein of a rabbit. The tests were carried out in the manner described in the previous paper, observations being made both macroscopically and microscopically in the small petri dishes whenever the two methods were applicable. The test antigen, except where expressly stated, was always the living organism suspended in a solution of peptone in 0.5 per cent NaCl.

Reactions of sera made with heat-fixed cultures

The work dealing with the heat-fixed cultures, the exudate and the washed bodies from which the exudate has been poured out can all be treated together.

Bresslau (1924) stated that if a culture of *Glaucoma* was centrifuged and washed and exposed to a temperature of about 35-36° C for $\frac{1}{2}$ -1 minute the organisms poured out an exudate which he named "Tektin". Further very rapid heating to 71-73° C caused fixation (heat coagulation) of the glaucoma bodies and solution of the exudate. If the material was now centrifuged a clear, faintly opalescent fluid was separated from the bodies. In practice this method required a little investigation and some further elaboration, but was carried out without any really material alteration.

The cultures used for this purpose should not be more than 48 hours old. The organisms were centrifuged down once, care being exercised to avoid as far as possible any damage or packing at the bottom of the centrifuge tube. St L was particularly sensitive to this while alive, and samples were rejected if any considerable number of dead and burst individuals could be seen under the microscope. Seventy-five c.c. of peptone culture were treated at once and resuspended in about 8 c.c. of 0.5 per cent saline. Two small water-baths were prepared, one at 39-40° C and one boiling vigorously at 100° C. The test-tube with the living organisms was fitted with a thermometer passed through a rubber cork and was held in the bath at 39° until the thermometer registered 34-35° C (usually for 1 minute and 15 to 30 seconds) and then was so manipulated that the temperature in the tube did not rise beyond 36° C for a further $1\frac{1}{2}$ minutes. The sample taken at this period and examined under the microscope showed living undamaged ciliates and a considerable amount of amorphous exudate causing a cloudy flocculent appearance in the tube. The tube fitted with the thermometer was then plunged into the bath of boiling water. Within 20 seconds the temperature usually registered 60°, a few seconds later as the mercury column passed the 65° mark the tube was removed from the bath. The temperature continued to rise to 73-75° and after a total of 1 minute 40 seconds from the moment of plunging the tube into the heat it was opened. The examination at this stage showed well-fixed glaucomas in fluid containing some cloudy material. The bodies were in good condition if the heating had not exceeded 78° and if the original rise in temperature had been sufficiently rapid, but a certain amount of shedding of clear hyaline discs or globules occurred without bursting or injuring the bodies. Their nature is not clear, but they disappear during the subsequent handling of the material. The still warm material was centrifuged again several times and a clear, faintly opalescent fluid finally obtained. The bodies together with some flocculent material remained as a deposit on centrifuging the heated contents of the tube.

produced the usual reactions, with however a slight alteration of the emphasis. Agglutination and the pouring out of exudate were well developed and appeared early, although the titre was not very high (1 160-1 320 for agglutination). Sheaths were also formed quickly, but were less solid looking and thick than those seen with a typical serum made with living organisms or with the unseparated "matrix". The sheaths were cast off rather rapidly and the agglutinated clumps dispersed, leaving the small plates in which they were observed filled with agglutinated masses of empty sheaths. These were still to be noted, though in reduced numbers and of shadowy appearance, at 1 640. In certain sera there was no detectable reaction at all, even in a concentration of 1 5, with the heterologous living strain Gl R. This was further tested and a very faint cross effect with Gl R. was detected in dilutions 1 5 and 1 10 of a serum made with a greater amount of antigen. These cross reactions were much more clearly shown in precipitation experiments dealt with later.

The sera made with the soluble exudate of Gl R. gave reactions which were along the usual lines, but the features were less distinctive than with similar sera made with St L. There was a slight cross effect with the living heterologous strain St L.

(3) The sera made with the washed bodies when used with the homologous organisms showed the familiar reactions but agglutination was delayed somewhat and, in the plates, developed rather poorly and irregularly, sheaths were formed in great numbers and on being cast off agglutinated into clumps and rafts.

While the distinctions were in no way sharply defined, there was a tendency in tests with antisera against the washed, heat-fixed bodies as compared with those in sera made with the supernatant fluid (soluble exudate), for the homologous organisms to show less complete agglutination in the plates and less free exudate. The sheath-producing effect on the other hand was very well developed, so that these shells were cast off after a time in such numbers that in dilutions of 1 160 and 1 320 the reaction involved practically all the organisms present and was the chief effect of the serum.

Reactions of sera prepared with acid-treated material

The antigens for these sera were obtained as follows. One hundred c c of culture (3-4 days' growth) were centrifuged and resuspended in 50 c c of 0.5 per cent saline, 50 c c of a 10 per cent solution of HCl in 0.5 per cent saline were added to the mixture and allowed to stand for 72 hours in the dark. The organisms were then washed five or more times in the centrifuge in saline. The pH of the suspension was tested and if found to be acid the washing was repeated. The rabbits showed no ill effects. The acid treatment fixed the organisms in the histological sense quite well. The method as also that used later for alcohol treatment is essentially the same as that used by Felix and Pitt (1936) for bacteria of the *Salmonella* group.

of these sera at 22 hours in dilutions up to 1 600-1 800 There were faint cross effects in the more potent of these sera, reduced as usual to the casting of a few sheaths on the part of the heterologous organisms

Reactions of sera made with steamed material

For the preparation of the sera from steamed organisms, the latter were grown in peptone water without sodium chloride and were centrifuged and suspended in neutral distilled water and steamed for 1-2 hours as desired Salt was added to bring the concentration up to 0.85 per cent after the heating was concluded

These sera had a very feeble action with the living organisms showing that the antigen had been very much altered by the treatment There was no agglutination of the ciliates and no immobilisation, but after 10-22 hours a certain proportion were seen to have cast their sheaths in dilutions up to about 1 40-1 80 and there was usually a little exudate shed into the surrounding medium Although these reactions were so very faint there was a just recognisable trace of cross effect with the heterologous strain Sera made with antigen steamed for 2 hours were even more feeble and a definite reaction could not always be registered in the experiments with the living organisms Table I gives the reactions to the various types of sera in tabular form

TABLE I

Reactions of sera prepared with various antigens

Antigen	Test object	Reaction
Living organism	Living glaucoma	Production of clear exudate, immobilisation, agglutination formation and casting of sheaths potent sera may cause death in higher concentrations
Soluble exudate shed into saline	" "	Exudate, immobilisation, agglutination, slight reduction in the formation and casting of sheaths
Washed bodies from which soluble exudate had been obtained	" "	Exudate, immobilisation and agglutination slightly reduced, emphasis is on the formation and casting of sheaths
Acid-treated	" "	Reaction as for serum from living antigen but titre much reduced
Alcohol-treated	" "	Exudate, immobilisation and agglutination much reduced, but formation and casting of sheaths well developed
Steamed for 1 hour	" "	No immobilisation or agglutination, only a slight degree of formation and casting of sheaths

treated antigen reacted poorly with the soluble exudate, but these were 18 months old when tested. Sera made with steamed antigen (1 hour) reacted with the soluble antigen and when tested with living organisms showed only the late sheath effect, the deduction being that a certain amount of sheath-forming substance was poured out into the saline as well as the clear exudate which seemed to be concerned with agglutination. This conclusion was borne out also as shown already by the effect of the serum made from the exudate.

Quantitatively the precipitin reaction could not be considered to give an accurate account of the amount of overlapping, as there was no means of standardising the antigenic content of the exudate solution and there was reason to suppose that the more prolific growth of Gl R probably yielded a greater amount of exudate.

Table II gives an abridged account of some of the precipitin reactions with various types of sera.

TABLE II

Abridged table of precipitin reactions with soluble exudate

Serum no. and antigen used in preparation	Precipitation reaction with						Experiment no
	Gl M soluble exudate			St L soluble exudate			
	1 2	1 4	1 8	1 2	1 4	1 8	
101 Gl M living	++++	+++	++(+)	+(+)	+(+)	+	P 91-2
96 St L living	++(+)	+(+)	+	+++	++	+	P 93-4
89 Gl M washed bodies	+++	++(+)	++	+(+)	+	(+)	P 95-6
88 St L washed bodies	+	+	(+)	+++	++	(+)	P 97-8
93 Gl M soluble exudate	++(+)	+(+)	+	+	+	tr	P 99-100
107 St L soluble exudate	+++	++	+(+)	+++	++	+	P 101-2
105 Gl M alcohol treated	++++	+++	++	+(+)	+	(+)	P 103-4
104 St L alcohol treated	++(+)	++	+(+)	+++	++	+	P 105-6
102 Gl M steamed 1 hour	++(+)	++	+(+)	+(+)	+	(+)	P 111-2
100 St L steamed 2 hours	tr	—	—	+	(+)	—	P 113-4
98 Hp 36 (Herpeto- monad)	—	—	—	—	—	—	P 43-P 90
Normal rabbit	—	—	—	—	—	—	P 53-P 76
Saline	—	—	—	—	—	—	

treated antigen reacted poorly with the soluble exudate, but these were 18 months old when tested. Sera made with steamed antigen (1 hour) reacted with the soluble antigen and when tested with living organisms showed only the late sheath effect, the deduction being that a certain amount of sheath-forming substance was poured out into the saline as well as the clear exudate which seemed to be concerned with agglutination. This conclusion was borne out also as shown already by the effect of the serum made from the exudate.

Quantitatively the precipitin reaction could not be considered to give an accurate account of the amount of overlapping, as there was no means of standardising the antigenic content of the exudate solution and there was reason to suppose that the more prolific growth of GI R probably yielded a greater amount of exudate.

Table II gives an abridged account of some of the precipitin reactions with various types of sera.

TABLE II

Abridged table of precipitin reactions with soluble exudate

Serum no. and antigen used in preparation	Precipitation reaction with						Experiment no
	GI M. soluble exudate			St L. soluble exudate			
	1 2	1 4	1 8	1 2	1 4	1 8	
101 GI M. living	++++	+++	++(+)	+(+)	+(+)	+	P 91-2
96 St L. living	++(+)	+(+)	+	+++	++	+	P 93-1
89 GI M. washed bodies	+++	++(+)	++	+(+)	+	(+)	P 95-6
88 St L. washed bodies	+	+	(+)	+++	++	(+)	P 97-8
93 GI M. soluble exudate	++(+)	+(+)	+	+	+	tr	P 99-100
107 St L. soluble exudate	+++	++	+(+)	+++	++	+	P 101-2
105 GI M. alcohol treated	++++	+++	++	+(+)	+	(+)	P 103-4
104 St L. alcohol treated	++(+)	++	+(+)	+++	++	+	P 105-6
102 GI M. steamed 1 hour	++(+)	++	+(+)	+(+)	+	(+)	P 111-2
100 St L. steamed 2 hours	tr	—	—	+	(+)	—	P 113-4
98 Hp 36 (Harpoto monad)	—	—	—	—	—	—	P 43 P 90
Normal rabbit	—	—	—	—	—	—	P 53-P 76
Saline	—	—	—	—	—	—	

TABLE III.
Reactions of homologous and heterologous strains of Glaucoma with absorbed and non-absorbed sera

Serum no	Antigen used to prepare serum	Material used to absorb serum	Strain used in test	Reactions and titre of serum with living glaucoma		No of exp
				Absorbed	Not absorbed	
68	Living Gl R	Alcohol treated Gl R	Gl R	Agglutination +(+), 1 1000 1 3200, no sheaths, 1 50	Agglutination +, 1 3200, tr, 1 6100 a few sheaths, 1 3200	113 and 414
68	" "	" "	St L	No effect, 1 12 5	Exudate and a few discarded sheaths, 1 100 1 200	105 and 406
70	Living St L	" "	St L	Agglutination (+), 1 3200, sheaths discarded and exudate, 1 3200	Agglutination +, 1 3200, sheaths discarded and exudate, 1 3200	110 and 112
70	" "	" "	Gl R	No effect, 1 12 5	Exudate and sheaths discarded, 1 12 5	109 and 411
68	Living Gl R	Steamed Gl R 1 hr	Gl R	Agglutination +, 1 6100, a few very shadowy sheaths, 1 800	Agglutination +, 1 6100, a few discarded sheaths, 1 3200	432 and 434
90	Washed bodies Gl R	Alcohol-treated Gl R	Gl R	No effect except tr of exudate, 1 20	Agglutination +, 1 320, sheaths discarded, 1 160-1 320	441 and 112
93	Soluble exudate Gl R	Alcohol-treated Gl R	Gl R	Agglutination +, 1 10-1 80, no sheaths, 1 20	Agglutination +, 1 1280, thin sheaths discarded, 1 610	443 and 444
105	Alcohol treated Gl R	Steamed Gl R	Gl R	Agglutination (+), 1 800, no sheaths, 1 200	Agglutination +, 1 800, a few sheaths, 1 3200	136 and 138
81	Alcohol-treated St L	Alcohol treated St L	St L	No reaction	Exudate and discarded sheaths, 1 10 1 80	382 and 384

with sera such as that made from the soluble exudate of strain Gl R and absorbed with the homologous alcohol-treated antigen

A typical experiment with this serum is shown in table IV. Here all the sheath-combining antibody was absorbed and the agglutination and immobilising titres were considerably reduced in the serum when tested without the addition of complement. When complement was added in a suitable dilution the death of about one half of all the glaucomas occurred, with complete lysis in a serum dilution of 1/40, the effect diminishing rather sharply with further dilution of the serum until only a trace was found in 1/320-1/640. The important point here is that the much weakened immune body consisted of the agglutinating and immobilising elements alone and when combined with complement death with lysis was produced.

In the unabsorbed control (expt 449, table IV), death increased in the dilutions from 1/40 to 1/1280 with diminution of the immune body, partial lysis appearing at 1/320 and a good degree of lysis only at 1/1280. With further dilution the number of dead diminished but those that were killed were also lysed. The sheath-reacting antibody apparently competed for complement or inhibited its union with the agglutinin.

Here again, as in the tests described in the previous paper, the lytic power of very small amounts of antibody when combined with complement is very striking, but it must be borne in mind also that the organisms exposed are alive and the action of a lytic agent on a small portion of the surface would probably suffice to destroy the complex balance of the peripheral system and disrupt the organism.

(2) The elucidation of the effect of the addition of complement to a serum containing only the sheath-combining antibody presented some technical difficulties also in getting a proper balance of complement and immune serum, and particular care had always to be taken to guard against the independent action of too large an amount of complement. The reaction was studied in sera made with steamed antigen and with alcohol-treated organisms. The results are shown in table V.

In experiments 340 and 343 serum 63 made with Gl R steamed for 1 hour showed no agglutination or immobilisation of the living homologous organisms and was completely innocuous as regards death, but produced the sheath-forming and discarding reaction in a moderate number of organisms in the later readings in a dilution of 1/5, diminishing to a trace in 1/20. In further dilutions there was no registrable effect. The addition of a suitable dilution of complement to the serum produced an interesting result. A proportion of the organisms were killed and agglutinated, but although the contents of the ciliates were sometimes half extruded

from the solid-looking sheath the substance of the more or less disrupted organism was in no way attacked or lysed. A rapidly diminishing number of dead were found up to a dilution of 1:40, still without lysis, and in further dilutions there was no effect at all. Here the sheath-forming antibody, by itself quite innocuous in this concentration, combined with complement to produce death with some degree of agglutination but without lysis. The effect has a perfectly simple relation to the dilution and is most pronounced in the highest concentration of the antiserum.

In experiments 297 and 301 (table V) a low-titre serum (81) made from alcohol-treated ciliates of strain St L was tested with the homologous living organism with and without complement. In this serum the sheath-combining antibody was present in sufficient amount to produce only the characteristic sheath-casting reaction up to a titre of about 1:80, while the agglutinin and the immobilising antibodies, which are always present to some extent in sera of this kind, were not powerful enough to be readily recognised in the reaction of the ciliates to the serum without complement. The addition of complement produced a combination with the sheath-reacting antibody in the higher concentrations which was lethal, killing large numbers of the organisms without lysis, while the trace of agglutinin and of immobilising antibody combined with the complement to produce a much restricted trace of lysis in 1:160-1:320 where the sheath-reacting antibody was not operative. Dilutions from 1:640 to 1:10,000 were without any effect.

Attention is drawn to the small amount of lysis (disappearing at the dilution 1:320 of the immune serum) correlated with the very slight development of agglutinin and immobilising antibody in this experiment. It is in sharp contrast to the very high titre of the lytic process in sera derived from the living antigen in the same circumstances.

Summary of the foregoing sections.

In those experiments where sera containing only, or little but, the sheath-forming antibodies were combined with complement, there was no increase in the lethal action in the higher dilutions and the effect disappeared with diminution in the concentration of the antibody. This seemed to be linked with the fact that there was little or no competition for the constant amount of complement between the two different types of antibodies. In the absence of the agglutinin and its allied elements there was no lysis and the sheath-combining antibody in conjunction with complement produced death but not lysis. Where, as in the absorbed sera, the agglutinins (using this to mean the antibodies derived from the

addition of complement to this antibody enhances its action in causing the death of the ciliates and the antibody interferes with the combination of complement with agglutinin (or antibody against the heat-labile elements), which alone is the active lytic agent in the immune serum

The division of the elements into heat-labile and heat-stable does not appear to be so sharp here as in the bacteria, for although antisera can be made with the steamed antigen they are of very low efficiency. The steamed glaucomas are capable of absorbing the sheath-combining element from the antisera, but it is very difficult if not impossible to remove all this antibody by this means from a serum made with the intact living organism

The conclusion is that these ciliates contain antigenic substances which share many of the characters of those already known in bacteria and that they contain both relatively thermostable elements analogous with the O or heat-stable antigen and more labile elements analogous with the H or heat-labile antigens of bacteria

The very active response of the surface of the organism is particularly striking and certain resemblances to bacteria suggest themselves, such as the marked swelling of the capsules of pneumococci when acted upon by a specific antiserum. Moreover the defensive mechanism of the sheath in these protozoa is perhaps of interest in connection with the greater virulence of certain capsulated bacteria (Topley and Wilson, 1936)

Summary

The antigenic properties of two varieties of ciliate belonging to the *Glaucoma-Colpidium* group have been investigated and sera prepared in rabbits from antigens obtained by treating the living glaucoma in various ways. From a study of the reactions of the living organisms to these sera, correlated with those found in sera absorbed by steamed and alcohol-treated glaucomas, it has been shown that the antigenic substances contained in these protozoa can be divided into two groups possessing different powers of resistance to heat (100° C for 1 hour) and to alcohol

Lysis of the ciliates results from the combined action of complement and the agglutinin evoked by the heat-labile antigen

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TABLE I
Myelosclerosis case 1. *Hematological findings*

Date	R B C (thousands per c mm)	Hb (per cent)	GI	Nucleated R B C (per 100 W B C)	Reticulocytes (per cent)	W B C (per c mm)	Varieties of leucocytes (per cent)										No per 100 white cells			Remarks
							Myeloblasts	N myelocytes	E myelocytes	B myelocytes	Proleucocytes	Polymorphs	Lymphophils	Basophils	Lymphocytes	Large mononuclears	Primary erythroblasts	Megakaryoblasts of Ehrlich	Normoblasts	
5 6 26	8,260	125	0 8			12,500						77	3	3	12	5				
28 12 26	3,300	62	0 9			18,700														
8 3 27	5,500	110	1 0			20,000						85	3 5	1	8 5	2				
9 5 27	3,810	75	1 0			22,000						70 5	5 5		14 5	0 5				
25 4 33	10,700	130	0 64			33,000						60	3		16					
17 5 35	3,550	58	0 82			27,000	15					57 5	13	4	17 5	3	0	5	1	
1 7 35	4,900	88	0 89			5,000	0 5	1	0 5		12	61	5	5	11	2	3	3	3	
30 7 36	3,300	60	0 9	18		18,000		2			0 5	55	31	7 5	2	2	3		33	
22 12 36	1,300	22	0 84	56		40,000	0	3			3	56	21	7		1	2	6	40	

and descending thoracic aorta, severe atheromatous calcification in abdominal aorta. Right common iliac artery, at point where it is crossed by ureter, showed a fusiform aneurism, $4 \times 3 \times 2$ cm, with considerable calcification in its walls. Other arteries of the body slight atheroma only. *Lungs* slight congestion and cedema of the dependent parts. Rest of the respiratory system normal. *Alimentary canal, biliary tract and pancreas, genito-urinary system* no lesion. *Liver*. 1945 g, pale but otherwise normal.

Bone marrow right femur only examined, whole marrow cavity red (fig 2), small portion only in upper third of shaft sufficiently free of bone for removal—it sank in water. The head and neck could not be examined.

Histology.

Spleen The architecture of the pulp was largely destroyed by fibrosis. This took the form of interlacing argyrophil fibres, just coarse enough to stain with van Gieson's stain (fig 3). The meshes were only wide enough to contain a few cells. Scattered through this fibrous background were numerous foci of hæmopoiesis, mostly erythroblastic but occasionally leucoblastic. The splenic sinuses were difficult to define. The malpighian bodies were small and very scanty. The vessels appeared normal and the trabeculae were not unduly prominent. Several typical Gandy-Gamna bodies were found.

Lymph glands One gland appeared normal, three others showed the following changes. The lymph follicles were relatively well preserved but the remainder of the gland had undergone a similar type of fibrosis to that seen in the spleen. In the fibrotic areas there was extensive hæmopoiesis.

Liver There were areas of hæmopoiesis in dilated liver sinusoids, the character of which was similar to that in the marrow. Erythropoiesis predominated and the cells tended to be immature. The portal tracts contained numbers of lymphocytes and a few immature red cells. The parenchyma showed slight brown atrophy.

Kidney In the interstitial tissue there were foci of hæmopoiesis similar to those in the liver. The parenchyma showed no significant change.

Adrenal The cortical cells were shrunken with rather scanty lipid. There were numerous foci of hæmopoiesis in the medulla (fig 4).

Ovary showed a few foci of hæmopoiesis.

Right iliac artery showed severe, calcareous atheroma and, at the site of the aneurism, destruction of the media.

Heart, lungs and pancreas showed no significant lesion.

Bones A radiogram of the femur taken after removal from the body on the same plate as a normal control (fig 5) showed irregular

genous marrow Further, several bays surrounding vessels indent the subperiosteal surface deeply or pass into subjacent medullary spaces or even pass through this compact zone to the deeper corticalis The bays contain a variable amount of hæmatogenous marrow, the hæmatogenous cells being closely packed in their deeper parts Beneath this more compact zone the corticalis in one relatively small area shows a compact bone rarefied by numerous Haversian spaces filled with hæmatogenous marrow Elsewhere the compacta is completely replaced by a small-meshed spongiosa, in which the more or less rounded medullary spaces are separated by trabeculæ which contain many systems of lamellar bone The spaces are packed with hæmatogenous cells The inner margin of this spongy corticalis is sharply defined, containing portions of a deep layer of inner fundamental lamellæ The medulla is occupied by a very fibrotic marrow composed of closely packed collagenous fibres and fibrils. It contains many granules of a yellow pigment which does not give the prussian blue or Turnbull's blue reaction for iron, nor Mallory's modification of the latter reaction Hæmatogenous marrow is sparse and forms streaks and patches in the fibrotic stroma It contains only fine small trabeculæ of bone

(2) *Longitudinal section of upper part of lower third of shaft* The corticalis is nowhere compact but in its outer part consists of large longitudinal medullary spaces separated by broad trabeculæ In its inner part the bone has been even more reduced by these spaces Close to the medulla the spaces become still larger, and from the spaces of the widely separated longitudinal trabeculæ there project laterally short, irregularly shaped, branching trabeculæ There is no sharp differentiation from the medulla, throughout which small trabeculæ are scattered at rather wide intervals Laidlaw's silver impregnation shows an astonishingly dense feltwork of stout reticulum fibrils The density is slightly less in the medullary spaces of the corticalis than in the medulla Hæmatogenous cells, scattered and in denser patches, are present throughout the medulla and become more densely packed in the outer medullary spaces of the corticalis One or two small medullary spaces immediately beneath the periosteal surface are free

The spongiosa

(1) *Head* With a hand lens the bone is seen to have lost the ordinary reticular pattern and to consist of groups of closely packed, small, irregular, anastomosing trabeculæ alternating with somewhat larger irregular areas free from bone The marrow is very fibrotic, but contains a few adipose tissue cells Hæmatogenous cells are scattered throughout but are unfortunately greatly shrunken

(2) *Inner condyle* Here the normal structure of trabeculæ

TABLE II
Myeloid leucosis case 2. Hematological findings

Date	R B C (thousands per c mm)	Hb (per cent)	GI	Nucleated R B C (per 100 W B C)	Reticulocytes (per cent)	W B C (per c mm)	Varieties of leucocytes (per cent)										No per 100 white cells			Remarks.
							My eloblasts	N my elocytes	D my elocytes	B my elocytes	Proleucocytes	Polymorphs	Eosinophils	Basophils	Lymphocytes	Large mononuclears	Primary erythroblasts	Megaloblasts of Ehrlich	Normoblasts	
10 10 31	6,000	70	7			18,000		0	5 5	1	0 5	31 5	3 5	1	51 5	0 5	1	+	+	Myelocytes present
12 2 32	4,800	60		6/200		3,750		2	4 5	0 5							12	1	1	Two transfusions
19 10 32	1,700	20	58	6/200		3,750		2	4 5	1	0 5	31 5	3 5	1	51 5	0 5	1	1	10	
3 4 33	5,400	74	5	42/200		13,300		1	4 5	0 5	1	38	5 5	4 5	40 5	2	1	11	16	
14 11 33	5,020	72	72	14/200	3 3	11,200		2	4 1	1	2	42	8 5	4 5	37	2	1	10		
8 11 34	4,240	68	76	23/200	7 3	17,500		2	5 5	1	1	14	6 5	8 5	27 5	55	7			
3 4 35	3,900	64	82	11/200	7 3	17,000		1	10	0	0	34	13	1	33	2	6	13	6	
27 2 36	3,200	60	93	22/200	9	10,200		1	14	3	3	37	2	1	13	1	9	13	1	
11 11 36	3,300	60	9	24/200	10	9,000			1	5	5	40	1	3	17	3				Drip transfusion Operation strangulated hernia
21 11 36	4,600	80	86			5,000														
4 5 37	2,900	49	84	13/200	5 4	8,400		2	3	1	1	53	4	1	33	2	5	1	4	
20 7 37	3,700	68	91	14/200	7 2	7,000		5		1	1	18	40	40		1	7	2	5	
16 9 37	4,200	74	88	7/200	3 5	9,000		0 5		3	3	28 5	3 5	6 5		18 5	7			
11 10 37	5,600	96	86	8/200	4 1	10,000		3 5		1 5	1 5	33 5	50 5		1	5 5	1	6	1	
20 9 38	4,100	60	7	7/100	4 4	22,000	1	4		1	1	50	30	6	1	7	1	3	3	

PLATE XXXVI

FIG 5 —*Case I* Radiogram of right femur (right) with normal control (left) after removal. Note the irregular inner surface of the corticalis, which appears invaded by streaks of marrow, and the irregular density of the spongiosa as compared with the control.

FIG 6 —*Case I* Radiogram of sections of shaft of right femur (right) with normal control (left). Note the decreased density and irregular inner surface of the corticalis and the patchy irregular character of the spongiosa compared with the controls.

phorus, bilirubin and phosphatase have been normal whenever examined, except that on one occasion the phosphatase was 14 units

Throughout the period of observation the spleen has varied in size considerably. It is always extremely hard on palpation. The patient has complained of sudden attacks of pain and discomfort in the splenic region but, apart from limitation of movement due to the rheumatic condition, remains in good health

DISCUSSION

The characteristic findings in the two cases described are (i) splenomegaly, (ii) irregular density of the spongiosa of the long bones and throughout the flat bones, associated with decreased density of the corticalis of the long bones, the inner edge of the corticalis being frayed and irregular in appearance, (iii) leuco-erythroblastic anaemia, (iv) increased red cell fragility in hypotonic saline, associated with increased cell thickness

Both patients were originally diagnosed as myeloid leukaemia on account of the large spleen and the immature white cells in the peripheral blood. More careful examination of the peripheral blood led to the recognition of large numbers of extremely young red cells which, in the presence of mild anaemia only, are suggestive of leuco-erythroblastic anaemia rather than of leukaemia. In neither patient could any source of a primary malignant growth giving rise to bone secondaries, the commonest cause of this type of anaemia, be found. Radiological examination of the skeleton in case I was negative 2 years before death but showed marked changes at autopsy. The skeleton of case II has shown during life similar abnormalities which appear to be progressive, and which radiologically are similar in character to those found at autopsy in case I.

The diagnostic significance of the increased red cell fragility cannot be assessed until further cases have been examined. It was associated in case I with an increase in cell thickness and in case II with a cell thickness on the upper limit of the calculated normal. Increased fragility is mentioned by Fiessinger and Olivier (1926) in a patient with leuco-erythroblastic anaemia and splenomegaly of unknown origin.

Case I showed an initial polycythaemia which appeared at the time to be of the Vaquez-Osler type. It is of interest that case II when first seen had a high colour and a red cell count of 6,000,000 per c mm, which though within normal limits is high, especially for a female.

A similar case showing leuco-erythroblastic anaemia and myeloid sclerosis following polycythaemia has recently been described by Stone and Woodman (1938). Cases of polycythaemia in which a leuco-erythroblastic picture with or without anaemia has occurred during the terminal stage have been described under the term

Both erythropoietic and leucopoietic tissues are involved and stress is laid in many reports on the proliferation of megakaryocytes (Rathery, 1902, Sternberg, 1904, Askanazy, 1904, Nauwerck and Moritz, 1905, Assmann, 1907, Donhauser, 1908, Levy, 1919-20, Cesa Bianchi, 1921, Goldschmid and Isaac, 1921-22, Firket and Campos, 1922, Barth, 1925; Fiessinger and Olivier, 1926, Ballin and Morse, 1927, Jaffé, 1927, Zadek, 1928, Dubinskaja, 1928-29, Speroni and Llambias, 1929; Gaudier and Houcke, 1930, Downey, Palmer and Powell, 1930, Wolf, 1932, Mizon (quoted by Émile-Weil, Chevalier and Sée, 1933), Carnot *et al.*, 1935, Hirsch, 1935, Tudhope, 1937, Hickling, 1937) Hewer describes his case as an example of megakaryocytic myelosis with osteosclerosis and likens it to those described by French authors as "*Splénomégalie myéloïde mégacaryocytaire amyelocythemique*" (Émile-Weil, Chevalier and Sée, 1933, Favre, Croizat and Guichard, 1934, Hugonot and Sohier, 1935, Weil, Isch-Wall, Perlès and Scemama, 1936) Whether these cells are true megakaryocytes of the type found in bone marrow is disputed. The picture differs from a typical leukæmia, firstly because cells of more than one type are involved, and secondly because there is dysplasia of osseous as well as of hæmopoietic tissue. The association of marked erythropoiesis and leucopoiesis with proliferation of giant cells distinguishes this group from that described by Stengel (1904), Wilson (1913), Kettle (1919-20) and Ross (1933), in which there was marked proliferation of giant cells only.

Some of the previous workers, in discussing the character of the pathological changes, have suggested that the leuco-erythroblastic hyperplasia is secondary to the sclerosis of the marrow, others that the sclerosis is secondary to the leuco-erythroblastic activity. Though no proof is at present possible we would suggest that both processes occur simultaneously in response to a single, at present unidentified, stimulus. All the cell types involved—osteoblast, fibroblast, hæmocytoblast and megakaryocyte—are derived from the same primitive mesenchyme reticulum cell of Maximow. It is noticeable that though the histological picture varies from case to case there is no instance recorded in which cells of the lymphocyte or monocyte type are involved. The nature of the postulated stimulus remains obscure. Stone and Woodman draw attention to the frequent occurrence of tuberculous foci in such cases. They were not found in the present (fatal) case nor in those previously recorded (Vaughan, 1936). Since such foci are not invariably present it appears difficult to attach to them any ætiological significance.

Apart from theoretical considerations it is important to recognise the existence of the somewhat ill-defined group of cases discussed above, because the duration of life is often longer than in typical leukæmia, and the prognosis given may therefore be better.

was therefore continued for a further two months, when the remaining eleven animals were killed. The results of this experiment were satisfactory in most cases but in some animals the vascular lesions were not severe enough for a difference between the two sides to be estimated satisfactorily. The experiment was therefore repeated using twelve experimental rabbits and twelve controls. In this second experiment an attempt was made to accelerate the production of cholesterol lesions by giving the cholesterol in a fatty base, in all other respects the procedure was exactly as detailed above. One gram of cholesterol was dissolved in 6 c.c. of hot olive oil and added to a small quantity of bran. The mixture was given in deep dishes to prevent spilling and other food was withheld till each animal had eaten its portion. By this means satisfactory lesions were obtained at the end of three months. In both sets of experiments the control animals received the same diet as the experimental but were not subjected to any operative procedure.

All animals were bled to death under ether anaesthesia. As soon as they were completely exsanguinated a cannula was placed in the first part of the aorta and the whole animal perfused with 10 per cent. formol-saline for ten minutes in order to fix and distend the vessels. The body was then eviscerated and a cannula was placed in the lower part of the abdominal aorta and the vessels of the hind limbs perfused, first with 50 per cent alcohol, then with a saturated solution of Scharlach R in 70 per cent. alcohol and again with 50 per cent alcohol. By this means the cholesterol lesions were stained bright red and their distribution could be mapped out with ease. This was done by drawing the lesions to scale on a semi-diagrammatic chart. After this had been done the main arteries of the hind limbs were dissected out from the bifurcation of the aorta down to the popliteal artery. A segment was then selected from each side for histology and frozen and paraffin sections prepared. The remainder was kept for chemical analysis. It was hoped that this would substantiate the differences in the distribution of the lesions noted anatomically. The vessels were stripped free from any attached fatty tissue and grouped into four batches—right and left sides of sympathectomised animals and right and left sides of control animals. The vessels were dried at 56° C. for four days and then extracted in a Soxhlet apparatus with chloroform for three days. The total dried chloroform extract was then weighed, saponified and the cholesterol precipitated as a digitonide.

During the course of the experiments all the sympathectomised animals were tested for differences of skin temperature between the two hind limbs. This was done by means of a thermo-couple and a string galvanometer, using a depilated area on the dorsum of each foot. On each occasion the temperatures were recorded three or four times at two-minute intervals. The tests were all performed at normal room temperature.

Results.

The results of the skin temperature tests are given in table I. In each case the figures represent the maximum difference of temperature recorded at that test. The difference of temperature varied from one animal to another and from time to time in the same animal, the mean difference being $3.77^{\circ}\text{C} \pm 1.93^{\circ}\text{C}$ (S.E.). In practice it was often found that when first removed from the cages the temperature of the two feet was high, with comparatively

little difference between them, but in the course of a few minutes, when the legs were extended for readings to be made, the temperature of the normal limb fell whilst that of the sympathectomised limb remained nearly constant. The limb on the sympathectomised side was almost invariably warmer than its fellow and was never less warm. This was taken as indicating that the operation had been successful in producing arterial dilatation during the course of the experiment.

In assessing the results of sympathectomy on the distribution of cholesterol lesions, the two experimental groups can be considered together.

Controls Among the eighteen control animals, one was killed early to ascertain if the treatment had been sufficient to produce the desired result and was found to have no lesions in the arteries of the limbs. The remaining seventeen all showed intimal cholesterol lesions on both sides. The severity varied from a few scattered plaques, mainly at the origins of branches, to severe confluent sclerosis. With one exception, there was no detectable difference between the two sides in either the extent or the severity of the lesions (fig). In this single exception, the lesions as a whole were scanty and were slightly more numerous on the left side.

Experimental animals. The eighteen sympathectomised animals showed lesions of about the same order of severity as the controls, i.e. varying from a few scattered lesions up to severe confluent sclerosis. In fourteen of these the lesions on the sympathectomised (right) side were more numerous than on the opposite side and in nine the difference was striking (fig). Of the four remaining animals one had no lesions in the vessels of the hind limb, two had only very slight lesions and the fourth had very severe sclerosis covering most of the intimal surface on both sides.

Microscopically the lesions showed lipoid infiltration of the intima with local proliferation of connective tissue. They differed in no way from those previously described by Anitschkow (1933) and therefore need no further description. The material was examined for any possible qualitative difference between the two sides but none could be detected, either in the intimal lesions or in the underlying media.

The results of chemical analysis tend to confirm the anatomical findings (table II). The cholesterol value for the right limb in the sympathectomised animals was 0.24 per cent higher than that for the left limb, whilst the difference between the two limbs in the control animals was only 0.1 per cent. The total amount of cholesterol present in the vessels was of a similar order in both experimental and control groups but the total fat values differed, being considerably greater in the control group. This is probably explained by the state of nutrition of the animals. In the sympa-

thectomised animals there was an average loss of weight of 152.2 g. whilst in the control animals there was an average gain of 59.4 g. It is probable that in the latter the amount of adipose tissue unavoidably left attached to the vessels was greater than in the experimental series, in which the animals were less well nourished

TABLE II
Cholesterol content of arteries

		Total fat (per cent)	Cholesterol as percentage of total fat	Cholesterol as percentage of total weight of vessel	Difference between two sides
Sympathectomised group	Right side	6.8	23.0	1.58	0.24
	Left side	6.2	21.5	1.34	
Control group	Right side	11.1	14.6	1.65	0.10
	Left side	10.2	15.2	1.55	

Discussion

The above results indicate firstly that lumbar sympathectomy causes relaxation of the affected vessels as evidenced by a rise of skin temperature and secondly that such vessels are more susceptible to intimal cholesterol sclerosis than normal ones. The main interest of this lies in its bearing on the findings of other workers and its possible bearing on the aetiology of human atheroma. The most closely related work is that of Danisch (1927-28) who showed that the occurrence of cholesterol sclerosis in the rabbit's aorta was accelerated by removal of the coeliac ganglion. Presumably the mechanism there is the same as in the present experiment. Schmidtman (1932) observed that in rats treated with cholesterol and vitamin D, enforced exercise in a treadmill gave rise to cardiac hypertrophy and an increased incidence of coronary sclerosis as compared with controls. Here it seems possible that a similar mechanism was at work and that the increased change in the coronary arteries was due to their being dilated in order to supply the overloaded heart. In a previous paper the same author (Schmidtman, 1929) had shown that cholesterol sclerosis can be accelerated by the simultaneous administration of vitamin D. Overdosage with vitamin D causes degeneration of the media with calcification and it seems possible that it was the weakness of the damaged media which facilitated the deposition of cholesterol in the intima. In this connection however it must be noted that if

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eggs, and in rabbits by intradermal inoculation or by scarification of the shaved skin

Strain G was isolated by Mr R. E. Glover of the National Institute for Medical Research, Mill Hill, in October 1937 from the scabs of typical lesions on an infected cow. During a period of two months this strain had been passed in the skin through five rabbits and subsequently through two rabbits by the testicular route. A piece of infected testicle from the last animal was received from Mr Glover and used as a source of virus in the present work. The material was kept at a temperature of 0°-4° C for four months before being used to inoculate developing eggs and guinea-pig pads.

Strain L was isolated by Sir John Ledingham in 1928 from fluid obtained from the vesicle on the arm of a milker who had been infected from lesions on the teats of cows on a farm in Somerset. This virus had been passed frequently in rabbits at the Lister Institute between 1929 and 1932. Between 1932 and 1938 infective rabbit material had been kept in glycerol in the cold and was then used to inoculate the skin of a rabbit. An elementary body suspension prepared from the skin lesions in a second animal by Dr C. Russell Amies formed the initial infective material used in these studies.

Strains of vaccinia virus

Strain A H was obtained from a sample of commercial sheep lymph. The lymph diluted 1:100 was used to initiate cultures on developing eggs and from the fluid subculture in series the virus was used to inoculate guinea-pig pads and rabbit skin.

Strain S. This was the Lister Institute vaccinia strain which had been passed repeatedly by scarification on the backs of rabbits and was supplied by Dr M. H. Salaman in the form of an elementary body suspension.

Strain A had been produced by passage through monkeys and rabbits of material from a case of human smallpox seen by Dr Amies in 1932. It was received from him in the form of dried elementary body suspension prepared from rabbit skin lesions.

Culture of virus on the chorio-allantoic membrane of developing eggs

Where the initial infective material was shown by culture on blood agar to contain bacteria it was suspended in broth and filtered through either a Berkefeld V candle or a gradocol membrane. Such bacteria-free filtrates contained sufficient virus to produce specific infections on the chorio-allantoic membrane of eggs. Eggs were used after preliminary incubation for 10-14 days and the technique described by Burnet (1936, pp. 9-16) was followed in making the inoculations. After 1-4 days further incubation at 36°-37° C, pieces of the infected membranes were fixed for histology or used to make smears and the remainder was rubbed up with a glass rod in 2 or 3 c.c. of sterile broth. After sedimentation 0.05 c.c. of the supernatant fluid, undiluted or diluted 1:100, was used to infect several fresh eggs and so cultures were carried on for many generations. Where it was desired to obtain single colonies falling tenfold dilutions in broth were used for inoculation.

Inoculation of guinea-pigs

The lesions produced by intradermal inoculation of virus into the plantar skin proved eminently satisfactory for histological purposes. When, as occasionally happened, signs of inflammation failed to appear within three days, re-inoculation usually resulted in a satisfactory "take". The animals were killed 3-18 days after infection and the pads and sometimes other

but owing to the lethal effects of the virus on the embryo in later passages, the fluid was diluted to approximately 1/100 from the 30th subculture onwards. Titration on eggs was carried out with the 2nd, 16th, 34th, 42nd, 49th and 65th cultures, and gave approximately the same titre in each instance, 0.05 c.c. of a 10^{-4} dilution giving from 5 to 30 isolated lesions. The 1st and 14th egg cultures produced typical lesions on intradermal injection into rabbits, while the 20th and 68th cultures were infective up to a dilution of 10^{-6} when tenfold dilutions were tested in the same way. The greater infectivity for the rabbit in these latter instances may have been due to the fact that the suspension was made by grinding the membranes in a mortar with sterile sand and buffer phosphate solution, whereas for titration on eggs infected membranes were rubbed up with a glass rod in tubes containing 2-3 c.c. of broth. Virus from the 18th, 26th, 27th and 54th cultures on eggs produced lesions on guinea-pigs' pads comparable to those produced by guinea-pig passage virus.

Appearance of the lesions. The lesions on the egg membrane showed the same characters throughout the serial cultures. With the large inoculum usually employed the membrane showed after 2 or 3 days' incubation a diffuse opaque thickening, frequently of reticular appearance, with extensive hæmorrhage into the membrane. Apart from the thickening and opacity due to ectodermal proliferation and cellular accumulation in the mesoderm, œdema of the mesoderm giving the membrane a thickened translucent appearance was common. In some instances large bullous swellings up to 1 cm. in diameter were encountered, due to collections of fluid between the mesoderm and endoderm. In older eggs it was not uncommon to find above the membrane a large collection of fluid which was sometimes clear but more often contained blood cells. When titrations were made so as to obtain single colonies, these appeared as discrete raised opaque spots up to 3 mm. in diameter with a central hæmorrhagic area. Fig. 1 shows the appearance of isolated lesions on 12-day eggs inoculated with virus from the 34th serial culture and incubated for 4 days more. These resemble the colonies described by Keogh (1936) for neurovaccinia. Secondary colonies were rarely encountered in egg membranes, even after 4 days' incubation.

Histology. Membranes examined after 24 hours showed extensive proliferation of the ectoderm, frequently localised. The mesoderm was usually œdematous but showed little cellular reaction apart from occasional proliferation of fibroblasts immediately subjacent to the thickened ectoderm. Small areas of hæmorrhage were sometimes seen. In sections stained by Mann's long method or with eosin-orange G and methyl blue small eosinophil inclusions were to be seen in the cytoplasm of the proliferated ectodermal cells, especially in the deeper layers, and in one instance in the cells of the mesoderm. These bodies varied in size from granules just visible with the

killed for examination the inguinal glands on the affected side were usually enlarged, congested and œdematous, but obvious lesions were not found in the internal organs

Histology The appearance of sections of the pad lesion after 3 days is seen in fig 3. The whole epidermis was greatly thickened and the needle tracks were usually recognisable just below the stratum corneum. Immediately below the needle tracks the greater part of the epithelial layer was replaced by coagulated fluid and fibrin, but this vesicular area was crossed by irregularly disposed strands of distorted epithelial cells. Around the central area the epithelial cells were enlarged, although vacuolation and ballooning degeneration were not commonly seen. Numerous sharply defined inclusion bodies with characters similar to those seen in infected egg membranes were present, especially in the deeper parts of the malpighian layer (fig 4) and also in the epithelial strands extending into the central area of œdema. In some cells a single large inclusion occupied the greater part of the cytoplasm, the nucleus being pushed to one side, while other cells contained as many as 7 or 8 smaller inclusions. At this stage the reaction in the dermis was usually slight. An increase of mononuclear cells and fibroblasts was usually seen around the vessels in the papillary zone and the endothelium of the vessels was generally prominent. Hæmorrhage extending into the malpighian layer was a common finding. Unless bacteria had been present in the inoculum, infiltration of the epidermis or dermis with polymorphonuclear leucocytes was not a feature of the lesion.

In pads examined after 6 or 7 days, areas of œdema in the epidermis had generally spread so that throughout the whole breadth of the pad the middle layer of the epidermis showed merely strands of cells and fibrin crossing the vesicular area. Hæmorrhage into the malpighian layers was always present at this stage and blood cells were to be found in the œdema fluid in the upper part of the lesion. The inclusion bodies were usually large and practically filled many cells in the malpighian layer. The nuclei of these cells, though often normal in appearance even when large inclusions were present, sometimes showed commencing pyknotic change. The vascular and cellular reaction in the dermis had increased. A certain number of polymorphonuclear cells were present in the region of the papillæ, although in the absence of bacterial infection infiltration with these cells was never striking. Inclusion bodies were not found in the dermis. In one animal killed 18 days after infection the œdema had disappeared but the epidermis was still markedly thickened. Slight infiltration with degenerated polymorphs was seen at a few points but no inclusion bodies were to be found. The dermis showed numerous fibroblasts and recently formed capillaries.

In the skin lesions the changes were of the same nature. Typical cytoplasmic inclusions were present in the greatly thickened epidermis and in the epithelium of the hair follicles. A superficial inguinal lymph gland from an animal killed 4 days after intradermal injection of virus into the flank showed marked œdema and congestion. In a few large macrophage cells at one point appearances resembling cytoplasmic inclusions were seen. In sections of spleen and liver from two animals no gross changes were apparent nor were specific inclusions identified. In one animal which had been injected both in the plantar skin and in the flanks, small papular lesions developed in one fore pad and on the upper lips, these were not examined histologically.

dermis The epidermis was markedly hypertrophied, the epithelium of sebaceous glands and hair follicles being also affected. The cells were frequently enlarged and in some instances there were spaces between them due to oedema. Numerous cytoplasmic acidophilic inclusions were present in the epidermis, particularly in the malpighian layer, hair follicles and sebaceous glands. These inclusions were similar to those seen in sections of infected egg membrane or guinea-pig pads. In the dermis hæmorrhages were always present, especially in the superficial papillary zone. The endothelium of blood vessels was unduly prominent and in the fibrinous exudate which occupied much of the central part of the lesion large phagocytic cells were commonly seen. Many of these cells and some of the endothelial cells lining thin-walled vessels in the dermis showed one or more large inclusion bodies. The central area of oedema with fibrinous deposit was usually bounded by a zone of marked cellular infiltration which extended to and involved the panniculus carnosus. In this area the reaction was similar to that described and depicted in vaccinal lesions in the rabbit by Ledingham (1924). Infiltration with polymorphonuclear cells was not a feature at this stage.

In sections taken after 6 or 7 days the epidermis in the centre had generally undergone necrosis and was infiltrated with polymorphonuclear cells. Numerous inclusion bodies could, however, be seen in the thickened epithelium bounding the necrotic zone. Hæmorrhage in the dermis was still a prominent feature and some of the vessels showed thrombosis. Cellular infiltration had increased, particularly around the vessels in the central zone of oedema and fibrinous deposition. As before, large inclusions were present in the cells throughout the central area and in a few sections inclusions were seen in histiocytes in the inflammatory tissue below the skin muscle. Polymorphonuclear leucocytes were never the predominant cells in the exudate but tended to be more numerous in the superficial layers of the dermis. By the 12th day the epithelium in the centre was completely necrosed and a dense zone of cellular infiltration was present, walling off the central part of the lesion in the dermis. After 20 days the centre of the lesion was being invaded by fibroblasts and the reaction around this area gave the appearance of granulation tissue. Inclusion bodies were still to be seen in large cells in the central area.

The lesions in the eyelids of certain animals following intradermal injection of virus were similar in histological appearance to those which developed in a rabbit infected by the intravenous route and are described later. In two of four corneas which showed opacity during life inclusion bodies were seen in localised areas of epithelial proliferation. The epithelial cells were slightly enlarged but there was little inflammatory reaction in the cornea. The inclusion bodies were few in number and smaller than those seen in well developed skin lesions.

Lesions produced by inoculation on the scarified skin of rabbits

As a rule no reaction was obvious 24 hours after cutaneous inoculation, but on the second day there was slight thickening and redness in the scarified area. After 3 days papular lesions had developed. These tended to be discrete rather than confluent, were of a bright red colour and on the 4th or 5th day were darker red and frankly hæmorrhagic. When vesiculation was observed the vesicles were very minute.

Histology In three-day lesions the epidermis showed marked hypertrophy, being frequently 6-8 cells thick. The cells were often greatly enlarged

cells Many of the mesodermal cells contained one or more inclusions and these were particularly numerous in fibroblast-like cells near the central cartilage of the ear In lesions of the eyelids specific changes in the surface epithelium were seen only on the palpebral conjunctiva, although in places the meibomian glands were involved in the inflammatory process Numerous inclusions were present not only in the greatly hypertrophic conjunctival epithelium but in scattered foci in the meibomian glands Sections of the spleen, liver and kidneys showed no specific changes

Cowpox virus, strain G

Material from infected rabbit testicle supplied by Mr Glover was used to initiate cultures on the chorio-allantoic membrane of developing hens' eggs The virus was carried through 36 serial passages in this way The naked eye appearance and histological features of the lesions on the membranes were exactly like those seen with strain B, the structure and distribution of the inclusion bodies being identical The infected membranes of the 4th, 10th, 20th and 22nd cultures on titration on eggs gave 5-30 colonies with 0.05 c.c. of 10^{-4} dilution and these had the appearance shown in fig. 1 The lesions produced by inoculation of the original material and of the 25th egg culture virus on guinea-pig pads were identical, on naked eye and microscopic examination, with those produced by strain B Figs. 5 and 6 are microphotographs of lesions produced by strain G on rabbit skin

Intravenous injection of strain G into rabbits

Two rabbits, G 17 and G 18, were injected intravenously with 1.0 c.c. of elementary body suspension which was infective in the skin in a dilution of 1:1,000,000 The suspension had been stored under ether for a few days and from 0.1 c.c. a few colonies of white cocci grew on blood agar Two further animals, G 67 and G 68, were injected with 1.0 c.c. of another elementary body suspension which was infective for rabbits in a dilution of 1:10,000,000 and on culture gave no growth of bacteria on blood agar Twenty-four hours after injection a small area on both flanks of each rabbit was shaved and that on the right side was lightly scarified

Rabbit G 17 developed conjunctivitis and 5 days after injection showed inflammatory thickening of the eyelids of both eyes and opacity of the right cornea No lesions were visible on the ears, flanks or lips, but next day the animal appeared very ill and was killed No obvious lesions were present in the internal organs Sections of the eyelids showed typical lesions on the conjunctival surface The cornea showed some degree of proliferation of the surface epithelium but specific inclusion bodies were not seen No definitely specific lesions were seen in sections of the other tissues or organs

Rabbit G 18 showed after 4 days a few red papules on the outer

Cowpox strain L.

This strain had been isolated in 1928 and was supplied in the form of an elementary body suspension by Dr Amies. The virus was used to infect egg membranes (4 passages) and guinea-pigs by pad inoculation. The lesions on egg membranes were identical with those produced by strain B and fig 2 was prepared from a section of the first egg membrane infected with this strain. The lesions on guinea-pig pads produced by the original suspension or by egg culture material were typical of these cowpox viruses. Rabbits were not inoculated with this strain but sections of the lesion produced in the skin of a rabbit by the original human vesicle fluid, kindly placed at my disposal by Sir John Ledingham, and sections of later rabbit material supplied by Dr Amies showed the same appearance as that produced by the other two cowpox strains.

The inclusion bodies produced by cowpox virus

In sections of infected tissue the cowpox inclusion bodies whether large or small showed no internal structure with the staining methods used. They stained pale homogeneous pink to bright red with hæmatoxylin and eosin, Giemsa, Mann's stain, or eosin, orange G and methyl blue, a deep red with acid fuchsin and methyl green and a blue-black colour, frequently with the margin shading off to a pale pink, with eosin-orange G and toluidin blue. With Heidenhain's iron hæmatoxylin the bodies stained uniform light grey to jet black according to the degree of differentiation of the section. No staining with Scharlach R was obtained in frozen sections after formalin fixation nor did the inclusions blacken in small pieces of tissue fixed in Gatenby's modification of Flemming's solution.

In smear preparations fixed with methyl alcohol prolonged staining with dilute Giemsa showed in successful preparations that the inclusion bodies contained numerous reddish to reddish violet elementary bodies which could best be seen around the edge of the inclusions. The finely granular structure of the inclusions could also be made out by examination of fresh unstained teased preparations or frozen sections by dark ground illumination. Free elementary bodies in vesicle fluid or in purified suspensions were readily demonstrated in wet preparations by dark ground illumination or in smears stained with alkaline methyl violet, in either case the free elementary bodies from cowpox material were indistinguishable from those of vaccinia.

Cultures on the chorio-allantoic membrane of hens' eggs.

Egg membranes infected with these three strains differed in appearance from those infected with the cowpox strains in that they never showed the hæmorrhagic character so typical of the latter virus. In all egg membranes with strains A H and A and up to the 20th culture with strain S, the membranes were frequently congested but did not as a rule show obvious hæmorrhages. After the 20th culture of strain S hæmorrhages were sometimes seen but they were never so extensive as in membranes infected with the cowpox strains. Collections of fluid above the membranes were infrequent and large bullous elevations were not encountered. Large single lesions were not so readily obtained because of the tendency to form smaller secondary colonies and the frequent generalisation of the takes after 3 days. Fig 9 shows the lesions on a 3-day membrane of the 21st subculture of strain S from an inoculum of 0.05 c.c. of a 10^{-5} dilution of the previous culture. The colonies tended to be less elevated, the edge was spreading and small daughter colonies were present. The dark hæmorrhagic centre characteristic of cowpox lesions was not a prominent feature. The virulence of the vaccinia strains was apparently greater, as indicated by the more frequent death of the embryo after 2 or 3 days and the spreading nature of the infection produced by high dilutions of virus. Titration of the 20th, 21st and 31st egg cultures of strain S showed good takes with 10^{-5} dilution and in the first two instances with the 10^{-6} dilution as well.

Histology The membranes infected with vaccinia showed much earlier degeneration of the proliferated ectoderm and ballooning degeneration with detachment of swollen cells was commonly observed. In sections of three day lesions the infected ectoderm had been lost in places, uncovering the mesoderm. Hæmorrhages in the mesoderm were seen especially in later cultures of strain S, but they were not so extensive as in membranes infected with cowpox virus. Infiltrating cells in the mesoderm frequently showed necrosis, and granular leucocytes were by no means uncommon in the exudate. The inclusions with all three vaccinia strains were alike and quite different from the sharply defined oval or circular inclusions of cowpox. They were usually confined to cells in the ectoderm, although in some three-day membranes mesodermal cells also showed acidophilic material similar to that seen in the ectodermal cells. The granular acidophilic material was sometimes present as a single mass at one side of the cell but was frequently distributed throughout the cytoplasm (fig 10 was prepared from a three-day culture after 34 passages of strain S). The inclusions appeared to be less strongly acidophilic than the bodies of cowpox and were more readily demonstrated with eosin-orange G and methyl blue than with Mann's stain.

Vaccinal lesions in guinea-pigs.

The inoculated pads usually showed an inflammatory reaction after 24 hours and after 48 hours the swollen pads were usually of a pale pink colour. By the 3rd or 4th day they had an opaque

of the lesions following intravenous inoculation of cowpox virus was like that observed by Camus in rabbits injected intravenously with vaccinia virus from calf lymph, and was much less extensive than that shown by neurovaccinia (Douglas, Smith and Price)

The inclusions produced by vaccinia virus

As noted above the inclusions produced by the three vaccinia strains appeared as irregular granular masses in the cytoplasm of infected cells quite unlike the large sharply defined homogeneous inclusions characteristic of the cowpox strains. That this appearance of the vaccinia inclusions was not the result of continuous rabbit passage was shown by the fact that strain A H on first isolation from commercial sheep lymph, produced the same type of inclusions. Under suitable conditions of illumination and magnification the acidophil granular masses could be resolved into numerous minute rounded granules which had the size and appearance of elementary bodies. Occasionally, larger uniformly staining bodies up to about $3\ \mu$ in diameter were present in addition to the granular masses. These bodies probably correspond to the "kugelige Körper" described and depicted by Herzberg (1936) and believed by him to be small aggregations of elementary bodies. Under certain conditions, however, vaccinal inclusions are commonly present in the form of regular evenly staining oval or round acidophil bodies (see Paschen, 1930). Such forms are typical of corneal infection in the rabbit and have been observed in vaccinal infection of the calf and in human variola and vaccinia, but it should be emphasised that such Guarnieri bodies are always much smaller than the larger cytoplasmic inclusions characteristic of the three cowpox strains used in this work.

It will be evident that although the general nature of the lesions produced by the two types of virus was similar, the differences were sufficiently constant and distinctive to allow differentiation to be made. On macroscopic examination the hæmorrhagic nature of the lesions caused by the cowpox strains was the chief distinguishing feature and on microscopic examination sections of vaccinal lesions showed a greater tendency to cellular necrosis, polymorphonuclear cells were more frequent in the inflammatory exudate and the inclusions were quite different in appearance from those associated with infection by the cowpox strains. In suitably stained sections the morphology of the inclusions was of itself sufficient to enable one to decide whether vaccinia or cowpox virus had been the infecting agent.

DISCUSSION

Observations made in the course of this work on the inclusion bodies produced by cowpox virus indicate that these, like the

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for the endothelial cells lining blood vessels. The lesions produced by the strains of cowpox on egg membranes and in the skin of rabbits bear a certain resemblance to those produced by neurovaccinia as described by Buddingh. Although no strain of neurovaccinia was used in the present work the records of Buddingh and others indicate that, in contrast to the cowpox strains, neurovaccinia is more virulent for chick embryos and for rabbits, its lesions are more frequently accompanied by cellular necrosis, and the type of inclusion is the same as that seen with dermal strains of vaccinia.

During the time the three cowpox strains have been under investigation there has been no tendency towards transformation to vaccinia, the character of the lesions and the appearance of the inclusion bodies have remained constant. The possibility remains, of course, that on more prolonged propagation of these viruses under laboratory conditions such change might occur.

The serological investigation of these viruses has not yet been completed, but the evidence at present available suggests that the differences indicated by the comparative study of the lesions are associated with certain differences in the antigenic make-up of these cowpox and vaccinia viruses.

SUMMARY

The lesions produced by three strains of cowpox virus on the chorio-allantoic membrane of developing eggs, in guinea-pigs and in rabbits, are described and compared with lesions produced under the same conditions by vaccinia virus.

Egg membranes infected with cowpox virus showed extensive proliferation of ectodermal and mesodermal cells, hæmorrhage and œdema in the mesoderm and the presence of large, compact, acidophil cytoplasmic inclusions in the cells of ectoderm, mesoderm and occasionally endoderm. Infected guinea-pig pads and rabbit skin showed œdema and hypertrophy of epidermis and hæmorrhage in the dermis. In the proliferated epidermal cells in both animals and in mesodermal cells in the rabbit, inclusion bodies of the type seen in egg membranes were always present. Secondary lesions in the skin, mucous membranes and testicles of rabbits infected by the intracutaneous or intravenous route showed the same characters as those seen in primary skin lesions.

In vaccinal lesions cellular necrosis was more marked and occurred earlier, while hæmorrhage was not a conspicuous feature. The inclusions appeared as irregular acidophil granular masses in the cytoplasm of infected cells, they were much less frequently seen in fibroblasts or other cells of mesodermal origin.

The difference in the form of the inclusion body associated with the two types of virus infection is discussed in relation to the differences observed in the histology of the lesions.

suggested by von Prowazek (1905, 1912, von Prowazek and Miyaji, 1914-15) It is the only tenable one at present, in view of the work of Goodpasture and his collaborators There are however two forms of this theory One of these supposes that the inclusions are intracellular colonies of the elementary bodies. The work of Woodruff and Goodpasture (1929, 1930) had shown that this was almost certainly true of fowlpox virus, Herzberg (1933-34) demonstrated that it was true for canary-pox, and Bedson and Bland (1932) and Bland and Canti (1935) proved it for psittacosis By inference therefore it was natural to assume that the vaccinia inclusion body was of the same nature

Other investigators have contested this theory Haagen (1937, Haagen and Kodama, 1937) and Herzberg (1936 *a* and *b*) both claim that in vaccinia the elementary bodies multiply diffusely throughout the cell Haagen considers that their presence in the inclusions is accidental and that they are found there only because they occur in any part of the cytoplasm The inclusion body cannot, he says, be regarded as a virus colony Herzberg takes a less extreme view He thinks that the inclusions are a form of virus colony and that the elementary bodies are an integral part of them At the same time he believes that this type of growth is not a necessity for the virus and considers that both the diffuse and the colonial form of multiplication can occur simultaneously in the same cell We believe both these theories to be erroneous We consider that the evidence we shall describe in this paper re-establishes the original theory of von Prowazek on a new and firmer basis and shows that the inclusion body is an integral stage in the life history of the virus

TECHNIQUE

Contemplation of the history of the controversy we have just described convinced us that it could only have persisted so long through failure to apply to the problem a technique adequate for its solution Bland and Canti had demonstrated the possibility of observing the process of intracellular development and multiplication of a virus in the living cell and Nauck and Robinow (1935-36) had shown that the inclusion bodies of vaccinia can be readily studied in cultures of rabbit's cornea Accordingly we set ourselves to combine these two techniques in the study of vaccinia We soon found, however, that the early stages in the formation of the inclusion bodies were so nearly invisible in the living cell, both by dark ground and direct illumination, and so difficult to distinguish with certainty from other cytoplasmic structures that we had to abandon the attempt We were thus forced back on some method of studying the process in stained specimens The work of Bedson and Bland had shown the vital importance in studies of this kind of relating the morphological findings in fixed preparations to the intervals of the dynamic time-sequence of the process of infection We considered therefore that any method we adopted must first of all enable us to date with accuracy the actual moment of infection of the cells and then to make a series of comparable preparations at various intervals

extremely transparent—a point of importance in dealing with whole mounts in which the cells are mostly somewhat thicker than in smears. (b) It stains both elementary and inclusion bodies. (c) It gives a colour contrast between the cytoplasm and cytoplasmic granules, which stain blue, and the elementary and inclusion bodies, which stain in shades of reddish purple. Upon this depends the possibility, extremely important for this work, of recognising elementary bodies when only very few are present in a cell. It is important also for the recognition of inclusion bodies, since there are some forms of these so diffuse that when stained with Victoria blue they cannot be distinguished from local condensation of cytoplasm. Their pink colour with Giemsa's stain at once reveals their true nature.

Counting experiments.

The examination of a very large number of preparations from experiments made by this technique showed that infection of corneal cells with vaccinia virus in this way is followed by the formation of a variety of types of inclusion body which we shall presently describe. These types succeed one another during the hours immediately following infection in a regular sequence so that at any given moment one particular type forms the majority of the inclusions present. While this preponderance of one type at a particular time is obvious at a glance it is not absolute and therefore we thought it advisable to have some numerical data on the subject. To obtain this we made what we call "counting experiments" in which each preparation of a series was counted through as in doing a differential leucocyte count on a blood film. The cells were thoroughly scrutinised for the presence of elementary bodies and the number of these and of the various types of inclusion body in each cell was noted. Counting was continued until 100 cells had been encountered containing one or the other or both, and at the same time a note was made of cells containing neither. The details of the technique are discussed in the appendix, the results are analysed in tables I and II. They form our chief evidence and are the basis of much that follows.

RESULTS

In addition to the formation of the specific inclusion bodies two other changes follow infection which may perhaps be termed semispecific, both of which have been described by Nauck and Robinow.

Breaking up. The first of these changes, the breaking up of the uniform sheet of cells into clumps and individuals, is caused by a contraction of the cytoplasm which results in the cells assuming fantastic shapes. Usually the main body of the cell around the nucleus is condensed into a narrow string which remains attached to the coverslip at its ends by broad fan-like expansions of cytoplasm (fig. 1). A similar phenomenon is sometimes produced by feeding cultures with a drop of fresh medium or tyrode, but the pattern produced is different. Thick cords and ribbons of cells are formed and the typical contracted cells with fan-shaped expansions are rarely found. Moreover such normal cultures rapidly spread out again in the course of an hour or so and re-form a uniform sheet. Infected cultures usually seem unable to do this and remain

PLATE XLIII

- FIG 1 —Breaking up of epithelial sheet into fan-shaped cells 1½ hours ×110
- FIGS 2a and 2b —Nuclear buds of (a) a homogeneous and (b) a fluffy appearance 1 hour ×930
- FIG 3 —Elementary bodies and a round object (arrow), slightly larger as well as more deeply stained than elementary bodies, in much flattened out cell Part of the nucleus is seen below 1½ hours ×1730
- FIG 4 —Apparent transition between elementary bodies and small homogeneous bodies in fan-shaped process of cell The two largest homogeneous particles proved to be Feulgen-positive 1½ hours ×1730
- FIG 5.—Small homogeneous bodies in much flattened out cell 1½ hours ×930
- FIGS 6a and 6b —Large homogeneous bodies, (a) stained with Giemsa's solution, (b) treated after Feulgen Nucleus and inclusions are seen to have given a positive reaction 4 hours ×930
- FIG 7 —Apparent transition between large homogeneous bodies and small networks, the latter fluffier and staining a lighter shade than the homogeneous bodies 2 hours ×930

All figures where not otherwise stated are photomicrographs of cells from rabbit cornea cultures fixed as whole mounts in osmic acid vapour and stained with Giemsa's solution The time given in hours is the interval between inoculation and fixation

little or no trace of purple. They often occur in company with the large homogeneous bodies, as in fig 7, and indeed all stages intermediate between these types can be found. We have found a single one as early as $1\frac{1}{2}$ hours after infection. The largest number found in a single cell is 21.

Medium networks (figs 8 and 9). These attain their maximum still later (table I: expt II, 3 and 4 hours, expt III, $5\frac{1}{2}$ hours, expt IV, 6 hours, expt V, 7 hours, expt VI, 10 hours). They are similar to the small networks but are larger, stain less densely and have a still more fluffy appearance. As with the preceding types intermediate forms occur (fig 8). The larger inclusions of this type are less homogeneous than the small networks and appear to consist of two substances, a slightly granular or reticular ground substance staining pink or red, and irregular lumps or rod-shaped masses staining deep bluish-purple. Often two or more of these medium networks appear to be fusing together to form a larger body (fig 9). They are often solitary and the maximum number we have found in a single cell is 7. The earliest we have found them is at 2 hours and they usually reach their maximum between 4 and 10 hours after infection.

Large networks (figs 10-14). These usually make their first appearance about seven hours after infection, though we have found a few as early as the third hour. They appear to increase steadily in number thereafter up to the twenty-fourth hour, by which time they attain their maximum (table I: expts II and VI). They are very similar to the medium networks and indeed the two types shade off into one another. They contain the same two types of substance, but the deep purple material, as well as occurring in lumps and rods, is sometimes split up into a larger number of small granules which may be no larger than elementary bodies, while the pink matrix is more diffuse and granular. These large networks may fill a large part of the cytoplasm and are usually found partially embracing the nucleus. The larger they are the more diffuse their ground substance appears, so that the largest are often hardly more dense than the cytoplasm. When, as sometimes occurs, they contain little or none of the deep purple substance, they are only to be distinguished from the cytoplasm by their pink colour (figs 11, 15a, 15b and 15c). This is the type of inclusion which we have said is difficult to identify in preparations stained with Victoria blue. Large networks are more varied in appearance than the other types. They are almost always solitary and we have never found more than two in a cell.

Study of table I, with the exception of expt VII, interpretation of which we must defer until we have discussed the relationship of the elementary bodies to the inclusions, shows clearly the sequence

PLATE XLIV

FIG 8 — Apparent transition between small and medium networks 2 hours
× 930

FIG 9 — Large medium network, suggestive of coalescence of three smaller forms
6 hours × 930

FIG 10 — Large network embracing nucleus Like the inclusion from the foregoing figure this network is seen to contain some granular material staining differently from the ground substance 15 hours × 930

FIG 11 — Large ill defined network to the left of the nucleus Groups of elementary bodies in the fringes of the cell 20 hours × 930

FIGS 12-14 — Examples of medium and large networks more highly enlarged than the foregoing seven figures to show varying appearance and arrangement of purple staining material embedded in the more or less homogeneous ground substance of the inclusion

FIG 12 — 15 hours × 1730

FIG 13a — Giemsa's stain 20 hours × 1530

FIG 13b — The cells of fig 13a treated after Foulgen. A positive reaction is given by the rod shaped material inside the large inclusion and by the transitional form between large homogeneous body and small network to be seen in the upper right hand corner An extremely faintly positive reaction is given by the matrix of the large inclusion The illustration is a rather inadequate representation of the delicate but distinct colour contrasts in the actual preparation 20 hours × 1530

FIG 14 — Large network containing long twisted lumps of purple material At a different level of focus and not visible here three elementary bodies could be seen in the periphery of this inclusion when studying the actual preparation 9 hours × 1530

of appearance of the various types. This is more clearly seen in the columns in which the figures are given for the number of cells containing each type than in those giving the total numbers, because the earlier appearing inclusions are more often multiple. Nevertheless the general drift from small homogeneous bodies to large networks is apparent in both. What is the reason for this drift? We consider that it is most satisfactorily explained by the assumption that the different types of inclusion are genetically related and that the small homogeneous bodies develop into the large networks by way of the other types which are the intermediate stages. This hypothesis is supported by the fact that nowhere can a hard and fast line be drawn between any of the types and that transitions can be found between them all. The process by which this growth occurs we believe to be threefold. In the first place genuine increase in bulk occurs, secondly there is a progressive decrease in the density of the pink component of the inclusions as shown by their increasing paleness and fluffiness, thirdly there is growth in size by coalescence. It is not possible to decide the relative importance of these three factors. Study of the figures given in the last five columns of table I suggests that up to the formation of the small networks coalescence does not play a very great part in the process of growth but that it is of considerable importance in the formation of the medium and large networks.

*Staining of the inclusions and elementary bodies
with Feulgen's stain*

Milovidov (1933-34) and Haagen and Kodama (1937) have stated that vacuolar inclusion bodies are Feulgen-positive. This we can confirm, but we find that a progressive decrease occurs in the depth to which the inclusions stain through the series of types. Both kinds of homogeneous body stain as deeply as the nuclei of the cells, the small networks are paler, while the medium and large networks become progressively fainter as they increase in size until a stage is reached when they must be regarded as Feulgen-negative. It will be recalled that a similar though less pronounced fading away occurs with Giemsa's stain and we prefer to ascribe it in both cases to a progressive decrease in density of the ground substance rather than to a change in its nature. Even in those large networks that are Feulgen-negative the deep purple rods and granules remain Feulgen-positive (figs. 13a and 13b).

Haagen has also stated that the elementary bodies are Feulgen-negative and this too we can confirm. Furthermore, they remain negative even when the modified technique is used by which Epstein, Ravich-Birger and Svinkina (1936) obtained positive results with tubercle bacilli.

TABLE II *Time relationships in the occurrence of elementary bodies and the various types of inclusion bodies in infected cells*

Experiment number and date	Hours after infection	No of cells containing elementary bodies				No of cells containing elementary bodies in association with the various types of inclusion body					
		Total number	Degrees of infection			IB	SH	LH	N1	N2	N3
			+	++	+++						
I 20 11 37	1½	55	49	6	0	3 2 1 0	51 47 4 0	1 1 0 0	0 0 0 0	0 0 0 0	0 0 0 0
	2½	7	7	0	0	0 0 0 0	6 6 0 0	4 4 0 0	4 4 0 0	1 1 0 0	0 0 0 0
II 27 10 37	1	24	19	5	0	19 15 4 0	5 4 1 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0
	2	2	2	0	0	2 2 0 0	2 2 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0
	3	2	2	0	0	0 0 0 0	0 0 0 0	1 1 0 0	2 2 0 0	2 2 0 0	0 0 0 0
	24	95	17	24	54	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	95 17 24 54
III 7 2 38	3½	23	14	8	1	3 2 0 1	19 12 7 0	2 2 0 0	2 1 1 0	0 0 0 0	0 0 0 0
	4½	1	0	1	0	0 0 0 0	1 1 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0
	5½	2	2	0	0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	2 2 0 0
IV 21 3 38	2	62	38	24	0	25 14 11 0	36 24 12 0	5 4 1 0	2 1 1 0	0 0 0 0	0 0 0 0
	4	0	0	0	0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0
	6	1	1	0	0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	1 1 0 0
V 24 1 38	4½	2	2	0	0	2 2 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0
	7	8	5	3	0	2 0 2 0	3 3 0 0	0 0 0 0	0 0 0 0	1 1 0 0	3 2 1 0
	11	48	17	19	12	3 1 2 0	1 1 0 0	0 0 0 0	0 0 0 0	2 2 0 0	42 14 16 12
VI 25 5 38	7	14	14	0	0	4 4 0 0	6 6 0 0	7 7 0 0	7 7 0 0	1 1 0 0	0 0 0 0
	10	3	3	0	0	0 0 0 0	2 2 0 0	2 0 0 0	0 0 0 0	2 2 0 0	0 0 0 0
	24	52	7	12	33	0 0 0 0	1 1 0 0	1 1 0 0	1 0 0 1	3 1 1 1	47 4 11 32
VII 30 8 38	4	0	0	0	0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0
	7	23	11	9	3	1 0 0 1	1 1 0 0	0 0 0 0	1 1 0 0	2 2 0 0	18 7 9 2
	11	27	10	8	9	0 0 0 0	2 2 0 0	0 0 0 0	1 1 0 0	2 0 2 0	23 6 8 9
	19	72	5	11	52	6 1 3 2	3 3 0 0	3 3 0 0	1 1 0 0	1 0 0 1	60 2 9 49

Contractions as in table I

+ = 1-6 elementary bodies

++ = moderate numbers of elementary bodies as in fig 16

+++ = large numbers of elementary bodies as in fig 11

In the last six columns the upper figure gives the total number of cells containing the given type of inclusion body in association with elementary bodies, the lower three figures indicate the number of cells showing each degree of infection with elementary bodies. Thus in expt I, at 1½ hours, column 8, there were 51 cells containing small homogeneous bodies and elementary bodies, and of these 47 showed a + infection, 4 a ++ infection and none a +++ infection.

PLATE XLV

FIGS 15a, 15b and 15c—Three different aspects of an indistinct large network containing groups and chains of elementary bodies together with several granules slightly larger in size, staining purple. (a) Outlines of large network indicated by black dots $\times 930$ (b) The interior of the cell in focus $\times 1730$ (c) The cell surface in focus, a few elementary bodies free in the cytoplasm $\times 1730$ 19 hours

FIGS 16a and 16b—Large network embracing the nucleus and apparently breaking up into groups of elementary bodies. The cell surface is in focus in (a) while (b) shows the interior of the cell and the marginal fringe. The latter is crammed with elementary bodies and bears a few "whiskers" with elementary bodies at their peripheral end. Maximow's Zenker-formol-osmic acid fixation, which may account for the coarse structure of the network. 20 hours $\times 1730$

Of these hypotheses we unhesitatingly prefer the last. By its very nature no evidence can be obtained either for or against the first. The second we consider improbable. Why should the elementary bodies which first penetrate the cell be destroyed while those which come later are able to persist? Why should those which reinfect the cells particularly penetrate those containing large networks. Moreover, by rinsing off the virus after five minutes' contact, we have taken steps in our experiments to reduce to a minimum the persistence of such free virus. The third hypothesis undoubtedly explains better than the fourth the common finding of a centrally placed network with elementary bodies in the cytoplasmic fringes. Nevertheless, it conflicts both with the evidence of Goodpasture, Woodruff and Buddingh that the inclusions contain masses of elementary bodies, and also with the cells, sometimes found in our own preparations, in which elementary bodies are visible in the inclusions.

The fourth hypothesis adequately explains the facts and it is in complete agreement with what we already know about some other viruses. The work of Woodruff and Goodpasture (1929, 1930) on fowlpox, of Barnard and Elford (1931-32) on ectromelia, of Herzberg (1933-34) on canary pox and of Bedson and Bland (1932) and Bland and Canti (1935) on psittacosis has shown that in all these cases the inclusion bodies contain numbers of virus particles. It is because it thus corresponds with what we know of the biology of other visible viruses that we prefer the last hypothesis to the third, which otherwise provides an equally adequate explanation of our findings.

We are now in a position to discuss the anomalous findings presented by counting expt VII. Here we find that at 4 and 7 hours conditions are as we should expect. At 4 hours small networks predominate, at 7 hours medium networks, if the figures for cells containing inclusions are accepted as the fairer basis of comparison. At the same time a number of cells with large networks containing elementary bodies has appeared at 7 hours. By 11 hours confusion appears to reign. The figures for cells containing small and large homogeneous bodies have again risen, medium networks have decreased and large networks with elementary bodies show a slight rise. By 19 hours all is once more as we should expect, the figures for cells with large networks and elementary bodies have doubled, those for the other types have been halved. We believe this experiment is very simply explained by the phenomenon of reinfection. With the reappearance of elementary bodies in association with large networks at the 7th hour the opportunity immediately arises for some of these elementary bodies to be set free from the cells and to reinfect others which escaped the primary infection. If this happened a rise in the

PLATE XLVI

FIGS 17, 18 and 19 —From cornea cultures with psittacosis virus showing colonies of large forms which mimic various types of vaccinia inclusions stained with Heidenhain's iron hæmatoxylin $\times 1730$

FIG 17 —Transitions between elementary bodies and large forms and small groups of the latter mimicking homogeneous bodies Compare with figs 4, 6a, 7 and 22 Culture 48 hours, in which reinfection has just begun

FIG 18 —Colony of large forms mimicking a small network. Compare with fig 8 Same preparation as fig 17

FIG 19 —Colonies of large forms mimicking medium and large networks Compare with figs 9 and 12 16 hours

FIGS 20a and 20b —Dark vacuole below nucleus in living cell (a) Fixed and stained with Giemsa's stain (b) By dark-ground illumination The vacuole proved to be a large homogeneous body Staining with Victoria blue and subsequent bleaching after Herzberg revealed the presence in the inclusion of 5 irregularly distributed granules, presumably corresponding to those seen inside the inclusions of figs 12-14 15 hours $\times 1340$

FIG 21 —Fan shaped process of cell showing homogeneous bodies some of which are rod- and dumbbell shaped, corresponding to von Prowazek's initial bodies 1½ hours $\times 930$

Relationship of our observations to those of our predecessors

It remains to attempt to relate our findings to those of other workers in this field. This is not always easy because of the variety of methods and materials which have been used, but for the assistance of the reader and the further elucidation of some aspects of our own work we append analyses of some of the more important items in the literature.

Ewing (1904-05) made impression (Klatsch) preparations of the rat's and rabbit's cornea which he dried in air, fixed by gentle heat and absolute alcohol and stained by Nocht's method. His photographs and drawings show that many fixation artefacts were produced, rendering the appearances difficult of interpretation in our terms. Allowing for these distortions his earliest changes seem to correspond to our small networks, and all succeeding stages are described. In the late stages he describes the appearance of a bluish material in the meshwork of the inclusion body, but it is doubtful if this corresponds to our deep purple material since the whole inclusion later became transformed into this substance. He made no attempt to relate findings to time after infection and he calls a given stage "early" or "late" merely because one is small and the other large.

Goodpasture, Woodruff and Buddingh made observations on the chorio-allantois of the hen's egg. The main importance of this work is the claim to have shown that the inclusion bodies contain masses of elementary bodies. The preparations which appear to clinch the matter are those in which the membrane was placed in 1 per cent acetic acid before making smears. In these (p. 277) "there are few if any dispersed Paschen corpuscles, but hyaline masses, for the most part compact, are present within and about the cells, and some of these are to be found smeared out thin enough to see that they are composed of the minute Paschen bodies." This agrees with appearances we have seen in some large networks. We have already said that our commoner finding of elementary bodies in the fringes and a centrally placed network apparently containing none may be due to the virtual identity of the staining properties of the matrix and of the contained elementary bodies.

Haagen and Kodama (1934-35) and *Haagen* (1937) found (i) that during the early hours after infection inclusion bodies may be found unaccompanied by free virus, (ii) that cells may be found containing elementary bodies but no inclusions, (iii) that both inclusion bodies and elementary bodies may be present in the same cell, (iv) that the inclusion bodies are positive, the elementary bodies negative with Feulgen's stain. *Haagen* interprets these findings to mean that multiplication of elementary bodies is independent of the formation of inclusion bodies and that the latter are of nuclear origin. Our evidence shows that at any time later than 7 hours after infection reinfection occurs and that, because of this, preparations made after this time will always show a mixture of forms. It is thus possible to interpret all *Haagen's* findings in terms of our theory but it does not seem possible for his theory to explain our findings. We reserve our opinion as to whether nuclear material plays any part in the formation of inclusions and we do not think that their positive Feulgen reaction is by itself sufficient evidence that it does. It is true that under normal conditions Feulgen-positive material is found only in the nucleus, but it is not legitimate to conclude from this that such material cannot be formed in the cytoplasm in response to the presence of a virus, nor that once within the cell the virus

may have overlooked inclusions in those cases where they were present without elementary bodies. Thus may be why he observed no typical inclusion bodies apart from the dark blue corpuscles, which we believe correspond with our homogeneous bodies in cells where Herzberg finds them in company with a few elementary bodies. Herzberg's cells filled to bursting with elementary bodies but containing no inclusions may well be cells with diffuse large networks, and his pictures confirm this, for in every case they show cytoplasmic condensations near the nucleus. The dark blue corpuscles he finds in such cells are perhaps granules of the dark purple substance we have described in our networks. On the other hand in a personal communication Herzberg states that in cornea cells he saw (1) small groups of elementary bodies, deeply stained like those in the egg membrane (? the deep blue corpuscles), (2) well delimited Guarnieri bodies free in the cytoplasm, (3) Guarnieri bodies extending crescent-like around the nucleus, (4) masses of free elementary bodies in the cytoplasm. This suggests that he is able to recognise some types of networks stained by his method and would have seen them if they occur in the egg membrane.

Himmelweit (1938) studied vaccinia lesions in the living chorio-allantois of the duck's and hen's egg by means of annular oblique incident illumination and high power objectives. He made no observations before the second day after infection, but because he studied the periphery of the lesions he considers that he was there able to observe the earliest stages of infection. We must point out that there is no guarantee of this. The true early stages which we have described may have been invisible by his methods and may have been situated still further to the periphery of the lesion than the zone in which he saw his earliest changes. Those who have read *Himmelweit's* paper may have been surprised by our statement (p 382) that the early stages in the formation of inclusion bodies were almost invisible to us, both by dark ground and direct illumination. By courtesy of Dr *Himmelweit* and Mr *Smiles* we have been able to examine some of our cultures infected with vaccinia and with psittacosis by their methods. It was thus possible to compare their microscopical technique with ours on familiar objects. We can say without hesitation that while their method gives slightly better definition than our dark ground system the difference is not striking and we do not believe that their method is capable of revealing structures invisible by ours. The tissue culture method enabled us to compare the appearance of structures in living and stained preparations in the same cell. Thus we have been able to ascertain precisely the appearance of some of our types of inclusion in the living state. Large homogeneous bodies, and presumably also the small ones, appear in dark ground illumination as optically empty dark structures having the same optical characters as the nucleoplasm. They reflect less light from their edges than the nucleus, are thus scarcely delimited from the cytoplasm and are not to be distinguished with certainty from other cytoplasmic vacuoles (figs 20a and 20b). Small networks have the same characters but even less well defined boundaries. Medium networks we have not identified with certainty. Large networks are large granular objects shining with a bluish-white light similar to that emitted by colonies of psittacosis virus (Bland and Cant) Their granularity is finer than that of psittacosis colonies, their whole appearance more "fuzzy," and they are less sharply delimited from the cytoplasm. An intense and rapid vibration of low amplitude is present within them. Accompanying the large networks we have seen accumulations of elementary bodies in the fringes of the cells and at the ends of the fine threads of cytoplasm already described (p 389). The appearance alive of these cells containing networks and elementary bodies corresponds precisely with *Himmelweit's*

corpuscles, usually double, staining deep red with Giemsa. These von Prowazek named initial bodies, we believe them to correspond to our small homogeneous bodies. He describes various division forms of these initial bodies. Some are dumbbell-shaped, some consist of two small bodies connected by threads, some are threads of various length and thickness. These appearances are difficult to interpret in our terms. We have occasionally seen such forms of small and large homogeneous bodies (fig 21) and more often of small networks, particularly when these are present in the drawn-out string-like processes of cells in "broken up" cultures. We think that probably most of von Prowazek's division forms are of this type. We do not agree with him that they are caused by division; our evidence suggests a more probable origin from coalescence. The first Guarneri bodies were found by von Prowazek at 3 hours after infection, as small round or oval bodies which take up nuclear stains. These correspond to our large homogeneous bodies. Still later, longer forms were found, corresponding to our networks, which consist of two substances, nuclear material containing chromatin (our deep purple substance) in the form of initial bodies and "plastin" staining red with Giemsa (our red matrix). Because of this and because of the presence of nuclear buds in his preparations, von Prowazek at first postulated a nuclear origin for the Guarneri bodies. Later he abandoned this theory. In his article of 1912 he describes the following findings: (1) elementary bodies both intra- and extracellular, (2) intracellular initial bodies; (3) transitional stages from these to Guarneri bodies, (4) Guarneri bodies in a variety of sizes and shapes with included initial bodies, (5) disintegration of Guarneri bodies and breaking up of initial bodies into numerous elementary bodies.

von Prowazek and Miyaji formulate their conclusions in final form thus: (1) elementary bodies invade the cell; (2) initial bodies are formed from them, often coated with the reaction products of the cell and in this state consisting of chromatin+plastin, (3) formation of Guarneri bodies with included initial bodies, (4) reappearance from these of elementary bodies. von Prowazek considered that this cycle of development was obligatory for the virus and did not believe in the multiplication of the elementary bodies throughout the cytoplasm. He proposed the name *Chlamydozoa* to describe viruses which undergo this form of development, a name which appears to us peculiarly apt in spite of the termination suggesting their protozoal nature, for the virus does most certainly wear a cloak, even a cloak of invisibility, during a part of its growth.

Tyzzar (1904) studied sections of experimental lesions of the cornea, skin and mucous membrane of the rabbit and calf. Fixation was in Zenker's fluid and the principal stain eosin and methylene blue. Tyzzar appears to have seen types of inclusion very similar to ours but his photographs are not sufficiently clear to permit comparison, while his camera lucida drawings, though resembling our inclusions in form, appear very different in structure. Tyzzar made an attempt to relate his findings to the time of infection. At 4 hours and 8 hours he found no inclusions but some were present at 16 hours. The majority of these were small round structureless bodies, 1μ in diameter, situated anywhere in the cytoplasm—obviously our small homogeneous bodies. Associated with these were similar but larger structureless bodies usually found near the nucleus. At later periods a mixture of forms was found, but, since the small bodies were always present at the periphery of the lesion while the larger were more centrally placed, Tyzzar thought the former the precursors of the latter. The later types were paranuclear in position, showed progressively more structure and contained granules staining like chromatin in a matrix staining like

Appendix.

Preparation of elementary body suspensions Through the kindness of Dr M H Salaman we have been able to obtain the strain of dermo-lapine that was used by Salaman (1937), Macfarlane and Salaman (1938) and Amies (1938), and which had already undergone 70 passages. While the present work was in progress we passed it on the rabbit's skin another 25 times. In preparing elementary body suspensions we followed the directions given by Salaman. Complete freedom from contaminating bacteria was not obtained. Nevertheless contamination of cultures was exceptional and such cultures were rejected.

Preparation of cultures The eyes were removed from a normal rabbit which had previously been killed by a blow on the neck. Each eye was gripped in sterile Lane's tissue forceps while 500-1000 c.c. of sterile saline was poured over it from a height so as to wash thoroughly the surface of the cornea and globe. With the eye held in Lane's tissue forceps and resting on sterile filter paper on a sterile glass plate, thin slices were cut from the anterior layers of the cornea with a sterile safety razor gripped in Spencer-Wells' forceps. Slices were cut as thin as possible so as not to penetrate the anterior chamber. These slices were cut into explants of about 1.5 mm. square and were mounted in a drop of heparinised rabbit plasma on a 20 mm. round coverslip fixed to a mica square coverslip as in Maximow's technique. A drop of rabbit spleen extract (6 c.c. tyrode to one chopped spleen) was added. The cultures were incubated at 37°C until growth of a sheet of epithelium had taken place and the medium had liquefied. This usually took 3 days. They were then either washed and fed and incubated for another 24 hours, or were used immediately.

Infection of cultures Various dilutions of virus were used for infecting the cultures—1:2, 1:3 or 1:4 for experiments concerned with the events of the first few hours only. The speedy and progressive disintegration of preparations infected with virus in this concentration made it necessary to use dilutions 1:10 to 1:30 for experiments meant to cover from 12 to 24 hours. In these cases disintegration begins at the periphery and very gradually progresses towards the more central parts, preserving the continuity of the epithelial sheet for the greater part of the duration of the experiment. Infection was carried out in the following steps.

The 20 mm. glass circles bearing the cultures were removed from the carrier mica coverslips. The liquefied culture medium was drained off to avoid uncontrollable dilution of the infecting suspension. Two drops of virus suspension in Tyrode solution were pipetted on to each of three cultures lying on several layers of filter paper in an ordinary Petri dish. After 5-8 minutes the infective mixture was drained off on sterile filter paper and for half a minute or so the coverslips were made to float, culture downwards, on warm saline solution. The dish containing the saline bath was gently rocked to and fro to ensure effective rinsing. The saline was again drained off and the cultures were supplied with a small drop of liquid medium (Tyrode extract of a broken-up clot of rabbit plasma and spleen extract) and remounted.

Fixation and staining 1. The cultures were made to float, culture downwards, for a minute or so on warm saline and exposed, three at a time, to a strong concentration of osmic acid vapour for 5-6 minutes. Shrinkage of the nuclei and an increase in the coarseness of all cytoplasmic structures occur if dehydration is left to the acetone-xylol mixtures which are employed to differentiate Giemsa-stained preparations. The cultures were therefore hardened in 70 per cent alcohol for 5 minutes or longer before staining.

the long horizontal camera by Zeiss, using the above-mentioned objectives in connection with an achromatic condenser N A 1.4, oil immersed, and Homal eyepieces by Zeiss

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Two possible methods of producing the polyuria were considered, firstly the method devised by Cushing (1932) of compressing the stalk of the hypophysis by a silver clip, and secondly the destruction of localised areas of the hypothalamus. The first method was discarded because (a) it promised to give little help in localising the nuclei concerned in water metabolism, and (b) it permitted an ambiguous explanation of the mechanism involved. Cushing has attempted to explain the resulting polyuria as the consequence of the obstruction to the flow of the antidiuretic hormone from the pars intermedia to the hypothalamus. As the studies on human material had tended to contradict this argument it was thought desirable to attempt to produce the syndrome by some other method. Finally in the hands of Mahoney and Sheehan (1935) the clip method had yielded only a transient polyuria.

TECHNIQUE.

The dog's head was placed in a clamp so that the base of the skull was uppermost (fig 1). Ether anaesthesia was maintained by an intratracheal catheter. A vertical incision was made in the temporal region by diathermy and the temporal muscle divided in the direction of its fibres and separated from its attachment to the bone. The periosteum of the zygoma was reflected and the zygoma removed. The tip of the coronoid process of the mandible was also removed. Whilst the temporal muscle was retained by two mastoid retractors the skull was trephined, and the trephine opening enlarged with bone forceps. Bleeding points on the bone edges were stopped with wax. The dura was then divided by a cruciate incision, the corners stitched back and the brain gently depressed by a spatula. The position of the head now allowed the brain to fall gently away from the base of the skull, and as gradual depression of the brain drove the cerebro spinal fluid out from the ventricular system it became possible to distinguish the hypophysis, the internal carotid artery and the third cranial nerve. Following division of the posterior communicating artery the tuber cinereum could be seen. In a few instances one optic nerve was sectioned in order to obtain a clearer operation field. After tearing the arachnoid and obtaining a dry operation field, small localised lesions 1.2 mm in diameter were produced in selected parts of the hypothalamus by passing the coagulating diathermy current along a copper wire insulated to within $\frac{1}{2}$ mm of its tip. The spark gap was closed almost to the point of short-circuiting in order to prevent gross local stimulation.

Before and after operation the animals were kept in metabolism cages. The urine was collected, and measured each morning. The specific gravity of the twenty-four hours' output was measured. Excess water to drink was provided.

After the periods requisite for study the animals were killed. In some, ammoniacal alcohol was injected into the skull, the brain with the hypophysis attached was removed and the hypothalamus and hypophysis fixed in ammoniacal alcohol and subsequently impregnated by Ranson's silver pyridine method. In others the brain was fixed in formalin and studied in paraffin sections stained by various methods. In all cases the hypothalamus was serially sectioned.

output, however, was replaced in sixteen animals by a second period of polyuria, the tertiary phase, which, once established, has persisted

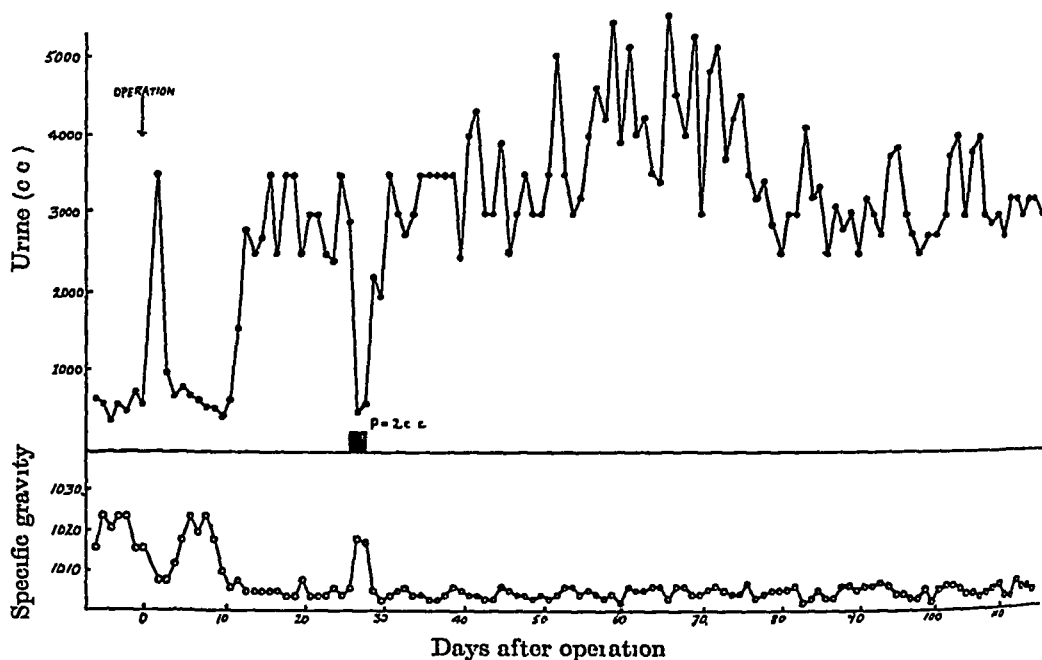


FIG 2—Graph of the urinary output in dog 13, illustrating the triphasic onset of the polyuria and the persistence of the third phase P = pituitrin 2 c c

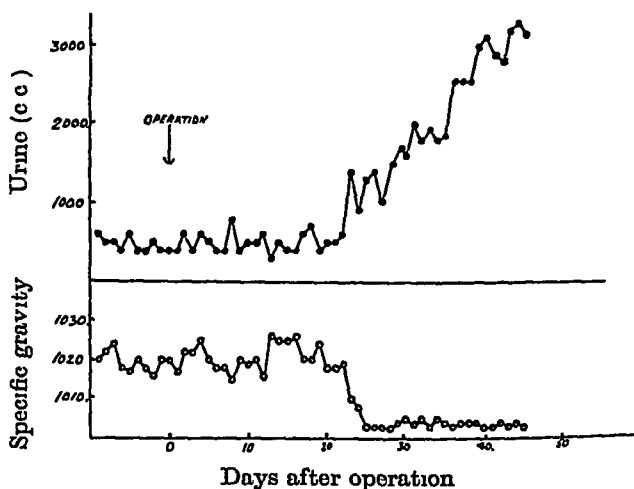


FIG 3—Graph of the urinary output in dog 43, illustrating the onset of the permanent polyuria without the occurrence of the primary phase

in all but two animals. In this group of sixteen the secondary phase lasted from 2 to 12 days (average 6.5 days). In four instances in which a permanent polyuria developed (dogs 24, 43, 45 and 48)

The third or permanent phase appears to be the experimental reproduction of a polyuria which simulates in every way the syndrome of diabetes insipidus in man. Apart from the polyuria the animals appear healthy and, though other changes may be found which indicate some disturbance in fat metabolism or in endocrine balance, there seems no reason why such animals should not survive to a natural old age.

Effect of pituitrin

In every animal in which a permanent polyuria developed, the effect of the administration of pituitrin was noted on the urinary output. Without exception this was found to reduce the polyuria of the third phase to within normal limits and to raise the specific gravity (fig 4). The administration of a second dose at a later date produced similar results. For example in the case of dog W (fig 4), 1 c c of pituitrin was sufficient to reduce the polyuria of 3000 c c to its normal output of 600 c c forty days after the operation. Two hundred days later a similar quantity had the same effect.

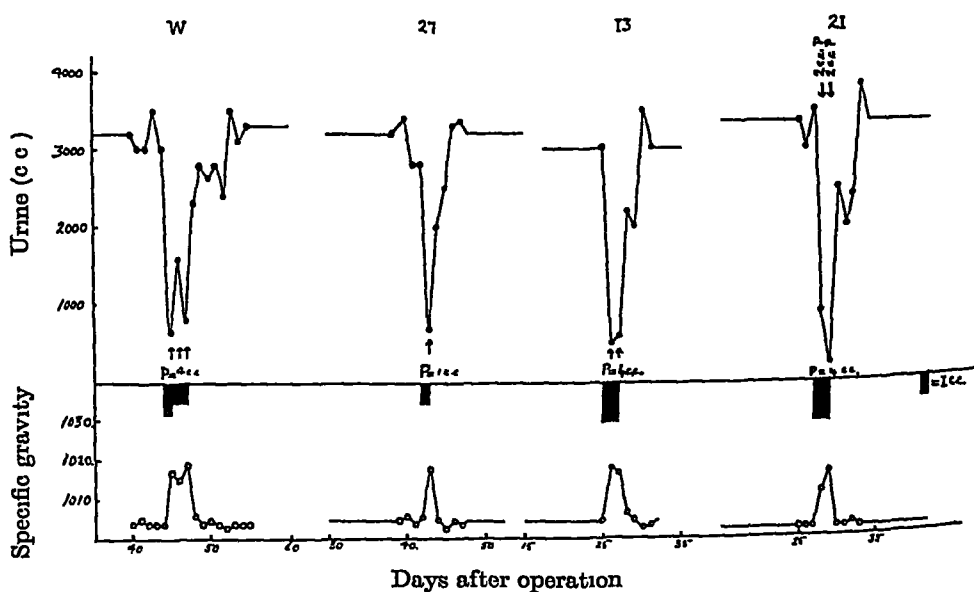


FIG 4—Graphs illustrating the effect of pituitrin (P) on the polyuria of the third phase

When, however, attempts were made to abolish the transient polyuria of the primary phase, the antidiuretic effect of the hormone was not apparent. The transient nature and indefinite period of the polyuria of the primary phase render it difficult to obtain comparable figures on successive days, but table IV shows the urinary output in this primary phase when dogs were given pituitrin sub-

A comparison of the results in a series of treated and untreated animals (fig 5) suggests that the primary phase is at least frequently refractory to pituitrin treatment

So far we have had no animals in which this refractory phase has persisted, and thus we have been unable to obtain any data analogous to those from human cases of the syndrome which are refractory to treatment (Biggart, 1937)

Effect of thyroid

Barnes, Regan and Bueno (1933) and Binsotti (1934) have shown that the thyroid possesses a diuretic action, whilst Mahoney and Sheehan (1935) were able to diminish the polyuria in diabetic dogs by thyroidectomy and to re-establish the increased urinary output by giving their thyroidectomised animals desiccated thyroid. They employed, however, extremely large amounts of the gland (2.5 g daily). Similar results have been reported by Fisher, Ingram and Ranson in their series of cats with diabetes insipidus.

We decided to give small amounts of thyroid by mouth. Thyroid (5 grains daily) was given to several normal dogs for six days, with absolutely no effect on the urinary output. When the same or even smaller amounts were given over a similar period to animals with diabetes insipidus an increase in the polyuria was frequently noted, commencing on the 4th or 5th day (dogs W, 13, 21, 36 and 38). The peak of the diuresis always occurred 2-4 days after the cessation of the thyroid feeding. The diuresis may continue for a long time even with these relatively small doses (dog W, 30 days, dog 21, 22 days, dog 13, 30 days). The sensitivity of diabetic dogs to thyroid may be gauged from the fact that a diuresis may be produced by 2-3 grains of the desiccated gland daily.

With the level of dosage used, diuresis has not been a constant finding. Dogs 24 and 33 showed no change in the urinary output, whilst in dog 27 a decrease occurred. This latter effect was so striking that when the urinary output had returned to its average level the thyroid treatment was repeated. On this occasion there was again some decrease in urinary output, though to a less marked degree than with the first course of treatment. In the first instance 30 gr of thyroid (5 gr daily) reduced a polyuria of over 3000 c.c. per day to a minimum of 1200 c.c. lasting for seven days. In the second instance a similar amount of thyroid reduced a polyuria of over 3000 c.c. daily to 1700 c.c. Following the treatment the curve of the urinary output was a mirror replica of that seen in the animals which showed an increased output, and we feel that the diminution of the polyuria was in this animal clearly related to the thyroid medication.

Effect of eucortone

Since it is generally agreed that the adrenal cortex has some control over the sodium chloride of the body, injections of eucortone

was allowed to live for a further 160 days, but apart from a slight fall for a period of six days after operation the urinary output continued unchanged

TABLE V
The effect of hypophysectomy

Dog	Normal daily output		Permanent phase of polyuria		Post-hypophysectomy output	
	cc	SG	cc	SG	cc	SG
27	600	1015	3200	1005	800	1012
38	300	1017	1280	1007	500	1013
36	510	1017	2030	1004	1750	1006

On killing these animals careful search was made for remnants of the hypophysis. The sella turcica and hypothalamus were serially sectioned. Apart from a few cells of the pars tuberalis embedded in the thickened meninges overlying the anterior hypothalamus no hypophyseal tissue was found in dogs 27 and 38. In dog 36, however, quite a large portion of hypophysis remained. The cells of this tissue appeared normal, and granule stains showed that both eosinophils and basophils were present.

Effect of oestrin

Injectations of oestrin have a very definite effect in inhibiting the activity of the anterior lobe of the hypophysis, at least as regards the formation of the gonadotropic hormone. It was considered that this physiological inhibition of the anterior hypophysis might give some additional evidence as to the function of this lobe in the syndrome. Two of our diabetic dogs were given 70,000 units of oestroform. In one a polyuria was reduced from 3300 to 2000 cc daily, but the effect only lasted four days. In the other no appreciable effect was produced.

Other effects of the hypothalamic lesion.

The dogs were weighed before operation and monthly thereafter. The results are shown in table VI. It will be noted that in many of those in which permanent polyuria was produced there was an associated increase in weight, amounting in some instances to more than 50 per cent of the pre-operative weight. At autopsy these animals showed a very heavy layer of subcutaneous fat. Fatty tissue was also present in abundance between the various muscle groups and heavy fat deposits were found in the abdomen. In the males there was marked atrophy of the testes, which histologically showed complete absence of spermatogenesis. The tubules were

pancreatectomy was performed on dog 46, 132 days after its primary operation (fig 16). This resulted in a dramatic disappearance of the polyuria, and though the animal was allowed to live for another 38 days the polyuria did not recur. Sensitivity to thyroid was also lost, and thyroid gr. 5 daily for twelve days failed to produce any alteration in urinary output. Evidently sufficient

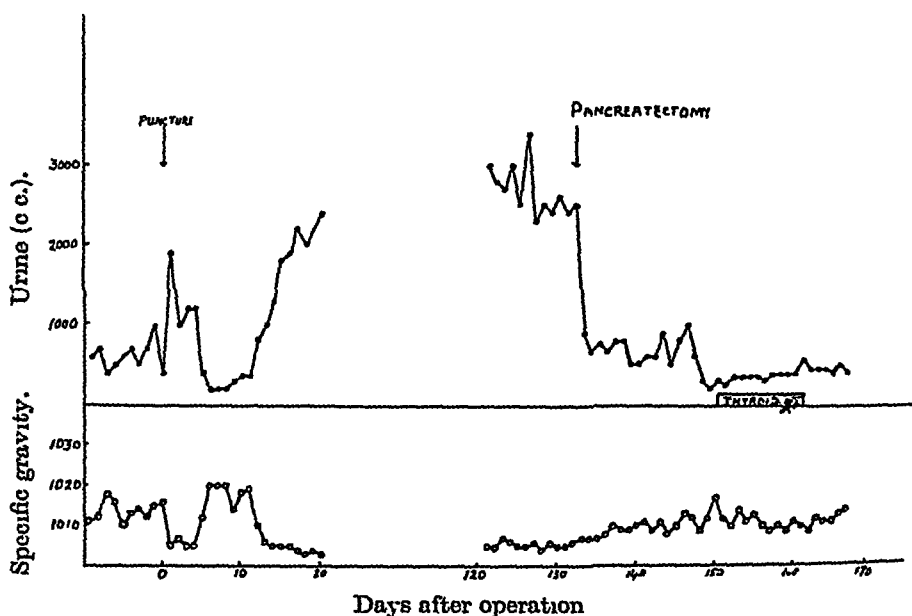


FIG 16 —Graph showing the effect of pancreatectomy (P) on the course of diabetes insipidus (dog 46)

pancreas was left to prevent any gross glycosuria, though the animal rapidly lost weight, losing 7 kg. in the 38 days. At autopsy a small fragment of sclerosed pancreas was found, but this measured only 1.5 cm. in length. The liver was extremely fatty, the other organs showed no changes of note. The median eminence had been completely destroyed.

DISCUSSION

It is apparent from this series of experiments that it is possible to produce diabetes insipidus in the dog by a lesion which involves the supra-optic nuclei or the supra-optic hypophyseal tracts. It is probable, therefore, that the failure of Reichert and Dandy to obtain similar results was due to the escape of these tracts or nuclei, but in the absence of histological studies it is impossible to state what nuclei were actually involved in their series of animals. The present histological studies also show that these hypothalamic lesions are associated with degenerative changes in the median eminence, stalk and posterior lobe, so that it is impossible to state that the actual mechanism involved is purely hypothalamic. It

phase of hormone deficiency or permanent polyuria. Whilst this appears to be a plausible interpretation of the results it cannot be accepted as completely proven. On this hypothesis complete removal of the secreting tissue should result in the abolition of the latent phase between operation and the appearance of the permanent polyuria. So far this has not been shown to occur. Fisher, Ingram and Ranson, who produced diabetes insipidus in cats by removal of the posterior hypophysis and stalk, found a latent period between the operation and the onset of the permanent polyuria in every way comparable to that following hypothalamic damage alone. Whilst it is probable that the difficulties of the operation preclude absolutely complete removal of the secreting tissues, it is surprising that the latent period should not have been considerably shortened.

The most difficult phase of the polyuria to interpret, however, is phase I. Here we have a polyuria which occurs even in the presence of a store of anti-diuretic hormone presumed to be sufficient to ensure a relatively normal output during phase II. Furthermore, injections of anti-diuretic hormone have in our series of animals failed to produce a return to a normal urinary output. This refractoriness of the primary phase to injections of pituitrin was also noted by Camus and Roussy (1913), who indeed state that the polyuria may be increased by such treatment. It is admittedly difficult to study the reaction of this phase to such injections. The phase may not occur even when no treatment is given, but we feel that its occurrence in spite of adequate treatment by pituitrin—the same pituitrin which effectively reduced the tertiary permanent phase—is sufficient evidence of this refractoriness. In our series of experiments the transient phase has occurred (1) in animals which subsequently developed permanent polyuria, (2) in animals which showed only a unilateral lesion of the supra-optic hypophyseal system, and (3) in animals in which no definite hypothalamic or hypophyseal lesion was found. Unfortunately the phase has always been transient and so it is impossible to state what morphological lesion if any is responsible. The transient phase has also failed to appear in animals which subsequently developed a permanent polyuria and which were found to show a bilateral lesion of the supra-optic hypophyseal tracts. It would appear, therefore, that the mechanism involved in this primary phase is not related to the nature of the permanent lesion in our animals. What the mechanism may be is not clear. On the basis of the morphological findings in human cases of diabetes insipidus refractory to pituitrin, it might be suggested that a post-operative oedema of other nuclei may set free diuretic mechanisms which are not controllable by the anti-diuretic factor. The points in favour of such a hypothesis are (1) the delay of 24 or more hours in the development of the polyuria,

this part of the hypophysis possesses a large reserve power as regards ability to produce diuresis. The fact that a portion of the gland is sufficient to maintain the diuresis is of some importance in human pathology in allowing us to understand how suprasellar cysts, which in many instances compress the anterior lobe and interfere with its function, can still continue to be associated with the syndrome of diabetes insipidus. It may also be the anatomical basis in those patients who, with the gradual progress of the lesion, show a diminution in the diuresis until the urinary output has returned to within its normal range. Such cases should be investigated from this standpoint.

Mahoney and Sheehan (1935) claimed that thyroidectomy completely abolished the polyuria of diabetes insipidus, but Fisher, Ingram and Ranson (1938) and Findley and Hembecker (1937) have shown that whilst thyroidectomy reduces it does not abolish the polyuria. Hence it would appear that the diuretic function of the pars anterior is not expressed solely through the thyroid. The inability of Dix, Rogoff and Barnes to produce a thyroid diuresis in a pancreatectomised dog suggested to us that the pancreas itself might act as a mediator of this diuretic function of the anterior hypophysis, and the result of pancreatectomy on dog 46 suggests that the pancreas is of no less importance than the thyroid as regards this function. In this dog it will be noted that thyroid administration failed to alter the urinary output after removal of the pancreas, whereas it was sensitive to thyroid in its polyuric phase. We believe, therefore, that the diuretic function of the pars anterior exists, but that the mechanisms whereby it is expressed are probably extremely complex and not dependent upon thyroid integrity alone.

The disturbances in fat metabolism and in the gonads following the hypothalamic lesion have previously been noted. In this series of animals the lesions were so placed that they interrupted any fibres from the nuclei paraventriculares to the hypophysis. Whether, as suggested by Greving (1928), these nuclei control fat metabolism it is impossible to say, but it seems probable that fat metabolism is controlled in some way by the anterior hypothalamic group of nuclei. This association of obesity with gonadal atrophy reproduces the clinical features of Fröhlich's dystrophia adiposogenitalis and suggests that this syndrome can be the result of lesions confined to the hypothalamus. Raab (1930) has tried to show that pituitary has some effect on lipid metabolism, and it is an interesting speculation that the control of fat may be by a neuro-endocrine mechanism analogous to that controlling water.

The mechanism of the production of the gonadal atrophy is difficult to understand. The anterior hypophyses of these animals appeared normal histologically. Both eosinophil and basophil

allow of a definite conclusion as to the precise site of origin of the hormone. The dramatic histological changes in the pars nervosa in our experimental animals seem to show that the pituicytes do probably play the important part in the elaboration of the hormone, and that the epithelial investments of this part of the hypophysis are not concerned. Studies of a recent unpublished case of diabetes insipidus in man show complete absence of the supra-optic hypophyseal tracts and atrophy of the median eminence of the stalk and posterior lobe, with greatly increased cellularity of these tissues. In spite of these changes the pars tuberalis is well developed. This is the second human case studied in which the histological changes found are in every way analogous to those seen in our experimental animals.

We conclude, therefore, that the supra-optic nuclei are in some way sensitive to the water needs of the body, that they serve as the nervous centres which stimulate the secretion of the anti-diuretic factor, and that this factor is in all probability manufactured in the pars nervosa. Diabetes insipidus results when this neuro-endocrine system loses its functional integrity, either by reason of a hypothalamic lesion interrupting the path of motor-secretory impulses, or by reason of replacement or destruction of the secretory tissue in the pars nervosa. In order that such loss of functional integrity may be clinically apparent a certain amount of functioning anterior hypophyseal tissue must be present. This diuretic function of the anterior hypophysis is probably mediated through other endocrine glands, and the evidence available suggests that the thyroid and pancreas are of importance in this respect.

SUMMARY

1 Temporary polyuria was produced in 29 dogs by the production of lesions in the anterior hypothalamus, and in 14 of these the polyuria was permanent.

2 The onset of this permanent polyuria is triphasic—a transient polyuria, a period of normal output, and finally the appearance of the permanent phase.

3 Hypophysectomy or pancreatectomy abolishes this polyuria, whilst thyroid medication usually increases it.

4 Lesions of the anterior hypothalamus may be followed by obesity and gonadal atrophy.

We are indebted to the late Professor Sir David Wilkie for the facilities offered by the Department of Experimental Surgery and to Professor A Murray Drennan for his helpful criticism. The expenses of the research were defrayed in part by grants from the Moray Fund to one of us (J H B). The other (G L A) has been in receipt of a part-time grant from the Medical Research Council.

with the findings of the author as published in a previous paper (Bisset, 1938) In the same paper the author indicated that the formation of colonies was influenced by purely physical factors

The aim of the present paper is to give some indication of the manner in which these forces act

OBSERVATIONS

The growth of bacterial colonies was studied primarily by the comparison of impression preparations made from colonies of different ages (Bisset) These observations were subsequently checked by observing the actual growth of two strains of vaccine charbonneuse, *B anthracoides* and an R and an S strain of *B coli* on an ordinary agar plate, in a microscope incubator and under the 16 mm objective of the microscope. Of these two methods the first was found to be of the greater value, as beyond a certain stage of growth the internal structure of an unstained colony is not easily observable, particularly if an organism is small

Smooth colonies

Very young S colonies of the coli-typhoid organisms were not entirely devoid of structure, but as they increased in age this appearance became lost or masked, until the characteristic, apparently structureless appearance was attained.

This was apparently due to the fact that the longitudinal attachment of the bacilli was so slight that a chain of more than ten or twelve became instantly distorted by the growth of the constituent organisms, hence the tendency to chaining was observable only in the very young colonies (fig 1) The break-up of such chains as might have occurred was accompanied by the "slipping" movement mentioned by Graham-Smith, Nutt, Seal and others, but in this case, i.e. on the surface of the medium, the slipping was merely a matter of the growth of the organisms after division resulting in the two opposing ends being pushed past one another until portions were overlapping, and sometimes until the bacilli lay side by side

A smooth colony in its early stages was seldom observed to be more than one or two organisms thick at the edge, with a "plateau" of greater thickness commencing about a third of the way to the centre, the dividing line between the two portions being quite sharp and distinct (fig 2) With increasing age the thickness appeared to increase somewhat, but usually remained a small fraction of the diameter, even in colonies which had the appearance of being strongly domed

Such smooth colonies also showed irregularities of outline corresponding to minor irregularities in the surface of the medium, and in growth threw out extensions in all directions, which gradually

after a prolonged lag phase, others in more heavily inoculated areas of the plate grew more readily, even though they were separated by as much as 20-50 μ from the neighbouring organisms

Rough colonies seldom reached any size without numerous off-shoots, consisting at first of a single bacillary thread or a very small number of such threads growing outwards from the edge of the colony. Such off-shoots, however, rapidly underwent the fate of the primary threads and eventually became mere lobes of the colony as it grew and advanced outwards

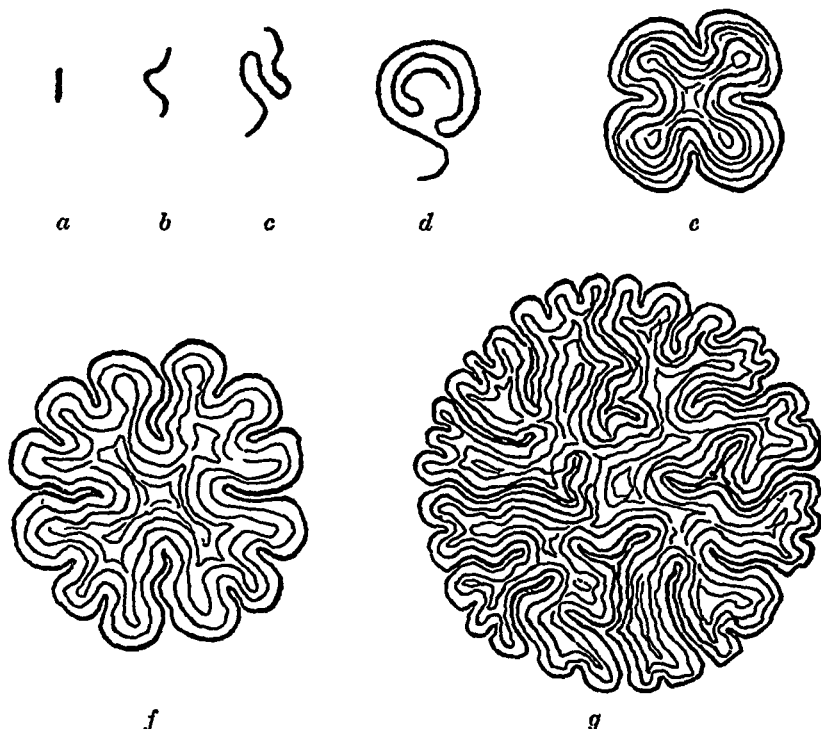


FIG 12 —Diagram of development of "medusa head" colony

a Original bacillus

b and c Extension and growth

d Primary coil

e, f and g Secondary and subsequent coiling

Except towards the centre of the colony the constituent threads of the coils did not appear to overlap one another to any extent and usually adhered firmly to the surface of the plate, under the influence of intermolecular attraction, so that the ultimate thickness of the colony was always, as in smooth types, a very small fraction of its diameter

The formation of the widely looped colonies of the streptococci differed in one respect from those of an R bacillary variant, in that the units composing the chains were so small that the chains themselves were much more flexible, thus frequently the loops were composed of a single chain and were consequently very numerous

In this species the first sign of division about to take place was the sudden appearance of an extremely fine line across the organism. This was followed in a few minutes by the appearance of a slight curvature of the dividing line producing a concavity on one side and a corresponding convexity on the other. Gradually the concave portion became convex also, until a progressive indentation appeared at the ends of the dividing line, thus completing the division. The whole process was completed in 5-15 minutes (fig 13a)

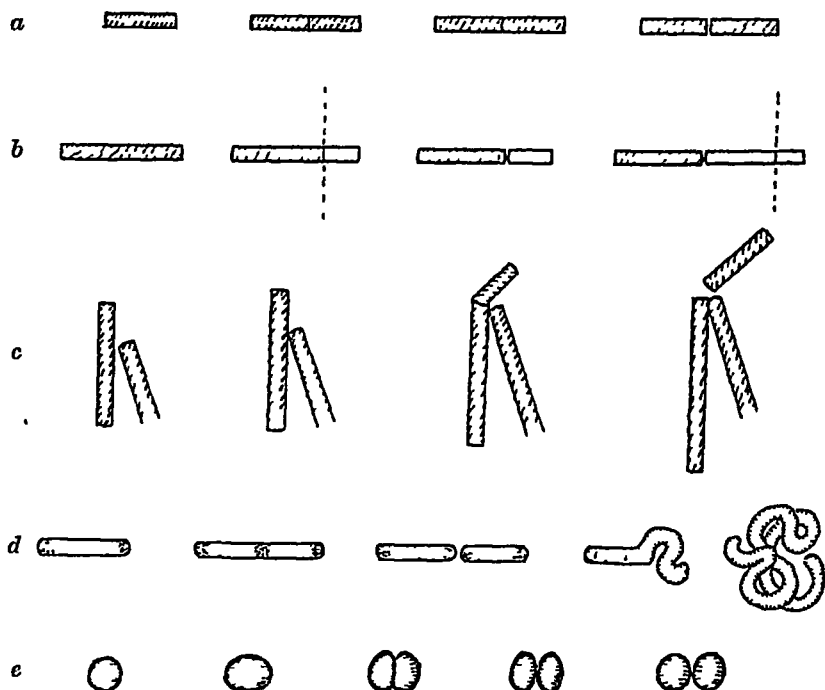


FIG 13 —Diagram of division of bacterial forms

- | | | | | | |
|---|--------------------------|-----------------------|---|----------------------------------|------------------|
| a | <i>Bacillus mycoides</i> | | d | <i>Vaccine charbonneuse</i> | Regular division |
| b | Anthracoid bacillus | Irregular division | | of S form | |
| c | Anthracoid bacillus | Division at point of | e | <i>Streptococcus hemolyticus</i> | |
| | | external interference | | | |

In the anthracoid bacilli, which were next studied, this sequence of events was often much less clear and was sometimes preceded by a swelling at the point of division, possibly indicative of a weakening of the envelope at that place, in others by a certain amount of constriction, forming a very narrow if occasionally deep groove around the bacillus. The behaviour of the S and R strains in this respect was distinguishable mainly by the greater tendency to constriction shown by the S strains, while the intermediate types were intermediate in this as in other respects. It was, however, most noticeable that the division of the S strains was into two equal portions, while the R strains usually divided into a large and a small bacillus, the smaller frequently growing so rapidly as to

the physical character of the individuals which compose them. These differences appear to be mainly in the strength and rigidity of their attachment to their neighbours in the colony, varying from practically nil in completely S forms to the very great rigidity of *B. mycoides*. In addition there are forms such as the long-chained *St. haemolyticus* in which the units of the chain are strongly but not rigidly attached. These qualities and differences are manifested in the colonial structure, under the influence of the external forces supplied by the surrounding material.

In the first place the bacteria are held closely to the surface of the medium by intermolecular attraction (the so-called "solid" medium being in fact a colloidal gel), and secondly the growth and consequent extension of the bacterial threads and chains across the surface of the medium is impeded by friction, which causes them to increase in length by the formation of loops and coils rather than by direct extension in a straight line.

Where the longitudinal attachment is very slight, as in S bacillary variants and short-chained streptococci, these loops and coils either do not occur or do so only to a limited extent—the bacteria being, for the most part, pushed past one another in the "slipping" motion as the chains elongate.

The observations of Nutt and of Seal on the modes of growth of R and S variants, which were made upon organisms growing in agar under cover-slips, were perfectly correct as far as they went. The application of these observations, however, made upon bacteria growing under conditions of severe mechanical restraint, to the same organisms growing in comparative freedom on the surface of an agar plate is not valid, and as far as these results are applied to surface colonies they are misleading. Graham-Smith, in his classical studies on bacterial growth, appears to have realised this, but did not take sufficient precautions to ensure the avoidance of this restraint.

In the case of an R strain growing under the first-mentioned conditions the bacillus would attempt to grow outwards and to elongate. Meeting with immediate opposition from the medium in which it was embedded, it would fracture at the point of division and produce the snapping growth described by these workers. In the same manner the two daughter cells resulting from the division of an S variant would be forced back past one another to lie side by side as soon as their growth approached the size of the original bacillus. Surface growth, however, is not restrained to the same extent, and the development of the colonies is as described above.

The studies on the actual division of the organisms presented a problem, inasmuch as while there appears to be a gradation in the complexity of the process, ranging from the elaborate septum

values fall within the normal range The blood findings are shown in table I

TABLE I
*Red cell fragility in tropical macrocytic anaemia **

Case no	Sex	R B C	H R	M C V	M C F	Corrected M C F
1	F	0 900	14 0	155 5	0 300	0 343
2	F	1 020	14 0	137 2	0 350	0 390
3	M	1 335	20 5	153 6	0 300	0 339
4	M	0 855	12 0	140 4	0 350	0 391
5	F	0 935	11 5	123 0	0 337	0 379
6	M	1 310	22 5	171 8	0 300	0 339
7	F	1 396	21 8	156 1	0 300	0 339
8	M	0 607	9 0	148 1	0 290	0 337
9	F	0 922	13 0	141 0	0 325	0 369
10	F	1 232	14 2	115 7	0 350	0 390
11	F	0 922	13 0	141 0	0 300	0 344
12	M	0 938	13 5	144 0	0 325	0 367
13	F	0 718	9 0	125 3	0 340	0 383
14	M	1 307	19 7	151 2	0 300	0 341
15	F	1 542	24 0	155 6	0 300	0 339
16	M	1 280	16 5	128 9	0 313	0 354
17	F	0 978	12 0	122 8	0 325	0 368
18	F	1 855	21 2	114 5	0 290	0 331
19	M	0 642	9 0	140 2	0 325	0 369
20	M	1 000	13 0	130 0	0 315	0 358
21	M	0 695	9 5	136 7	0 340	0 385
22	M	1 880	27 5	146 2	0 310	0 329
23	F	0 530	8 0	151 0	0 350	0 392
24	F	0 822			0 325	
25	M	0 895	12 0	134 1	0 262	0 310
26	M	0 945	14 5	153 5	0 255	0 302
27	M	0 822	11 7	143 0	0 357	0 398
28	F	1 187	15 5	130 6	0 375	0 428
29	M	1 835	24 7	134 9	0 325	0 343
30	M	0 618	11 5	186 1	0 325	0 367
31	M	0 750	11 7	156 7	0 315	0 358
32	M	0 866	10 0	115 4	0 300	0 345
33	M	0 822	13 5	164 3	0 285	0 330
34	F	0 835	11 8	141 4	0 340	0 382
35	F	0 583	10 0	171 5	0 340	0 382
36	M	0 582	8 5	146 1	0 315	0 359
37	F	1 270	13 2	104 3	0 320	0 362
38	M	2 010	23 0	114 4	0 288	0 310
39	M	2 695	35 0	129 9	0 290	0 308
40	M	2 960	38 5	130 1	0 300	0 316

* R B C = Red blood corpuscles (millions per c mm)
H R = Haematocrit reading
M C V = Mean corpuscular volume in μ^3
M C F = Median corpuscular fragility (g per cent NaCl)

Results and discussion

The value for the normal mean M C F used in the present work is that obtained by Dacie and Vaughan in a series of 50 healthy subjects In our series the mean M C F for the 40 cases was 0.316 ± 0.004 per cent NaCl, a figure significantly lower than the normal value of 0.366 per cent Further, the range of values found and the coefficient of variation were markedly increased (table II, and chart I) These figures, however, cannot be accepted as

expressing the true fragility, as part of the decrease is due to the higher proportion of plasma, and therefore of salts, in anæmic blood (Creed, 1938, Dacie and Vaughan, 1938), which causes a considerable increase in the salt concentration of the test solution. The amount of plasma added can be calculated from the hæmatocrit

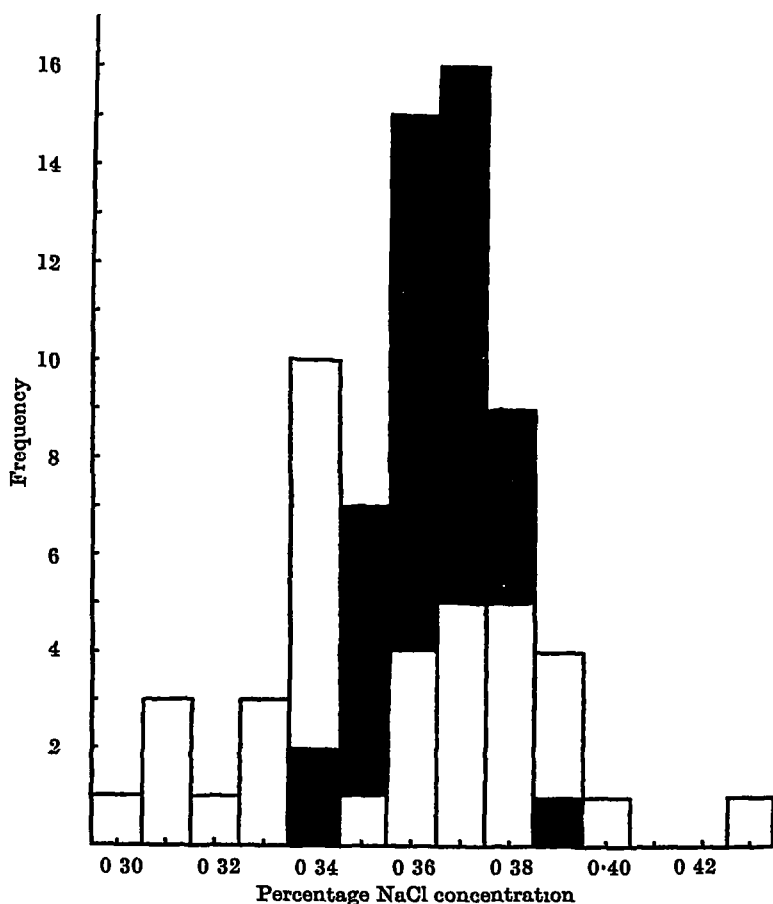


CHART 2—Frequency distribution of M C V

- Normals, ♂ and ♀, 50 cases (Dacie and Vaughan, 1938)
 □ Tropical macrocytic anemia, ♂ and ♀, 39 cases Corrected values

reading, and if the amount of saline used is 1 ml, i.e. 22 drops, the final concentration, assuming plasma to be equivalent to a 0.9 per cent NaCl solution, will be $\frac{22 \text{ M C F} - 0.9 \text{ P}}{22 - \text{P}}$, where "P"

is the amount of plasma added to each tube. The figures obtained in this series, thus corrected, are shown in the last column of table I and in chart 2. The difference between the mean corrected M C F and Dacie and Vaughan's figure for normal M C F is 0.010

importance is attached to this finding. The single case with a greatly increased M C F was a woman with a history of malaria and a greatly enlarged spleen, but no signs of recent malaria.

Summary

1 The uncorrected median corpuscular fragility in 40 cases of tropical macrocytic anaemia lay between 0.255 and 0.375 per cent. NaCl, with a mean of 0.316 per cent. The normal mean of 0.366 per cent, determined by the same method, is significantly higher.

2 The difference could be largely eliminated by applying a correction for anaemia: the range in 39 of the above cases then becomes from 0.302 to 0.428 per cent NaCl, mean 0.356 per cent. The normal mean is only probably significantly higher than this corrected figure. The exclusion of all pregnant or recently delivered patients from the anaemia series made the difference between the mean M C F of this series and that of the normal 0.0174 per cent NaCl, standard error 0.0068, a significant difference.

3 The M C F for men and non-pregnant women was significantly lower than the M C F of pregnant women but the number of pregnant women tested was small.

4 It is suggested that pregnancy may result in an increased fragility, masking the decrease which takes place in uncomplicated tropical macrocytic anaemia.

5 Using the corrected figures for M C F there was no correlation between the degree of anaemia and the value of this figure.

The expenses of this work were met by a grant to one of us (L. W.) from the Lady Tata Memorial Trust. We wish to express our thanks to the physicians of the Cama and Alpbess, the Golculdas Tejpal, the Jamsetjee Jeejeebhoy and the Bai Motlibai Hospitals, Bombay, who kindly allowed us to investigate their patients, and to Colonel S. S. Sokhey of the Haffkine Institute for laboratory facilities.

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demonstrate gross differences in bactericidal power in a striking manner (Fleming, 1938), it is not the method of choice for all purposes. It is somewhat misleading in that it fails to register the maximum bactericidal effort of which a given blood is capable. Evidence of this will be given later in this paper. It may be misleading also in that the failure to develop visible colonies in the blood may be attributed to actual destruction of the cocci, whereas in reality these are alive but unable to multiply. In practice such a mistake will only occasionally occur. The method is also inconvenient because the rapid development and coalescence of hæmolytic zones round the colonies make it necessary to observe the cells at intervals from 6 to 15 hours. This spreading hæmolysis usually makes it impossible to count discrete colonies after about 8-10 hours.

The agitated tube method

The infected blood is incubated in tubes and measured samples removed at appropriate intervals to determine the number of viable cocci surviving. To obtain an optimal bactericidal effect it is essential to keep the blood in constant motion, thus securing frequent contact between leucocytes and bacteria (Todd, 1927a). For this reason the method of "stationary" tubes is of very little value for bactericidal experiments with blood and need not be further considered.

In almost all the experiments here reported the tubes were agitated, either by rotation on a drum (rotating tube method of Todd, 1927a), or by frequent contact with rubber vanes revolving in a water-bath at 37° C (water-bath method of Willcocks, 1938). This procedure is unsatisfactory in one respect, namely, that it usually involves making a bacterial count at least twice in the course of incubation if we are to obtain an instructive record of the changes occurring in the streptococcal population.

With human blood this population usually shows a rapid decline during the first 1-3 hours, owing to destruction or aggregation of the microbes by the leucocytes, after that time (depending upon the conditions of experiment and other factors) any streptococci which have survived multiply more or less rapidly, often reaching a maximum of many millions per c.c. within 24 hours. All depends, therefore, upon the time of making the bacterial counts whether we register an incomplete or a maximal bactericidal effect, or none at all. We know of no way in which this difficulty can be overcome. In spite of it, the method appears to us to give more valuable information than any other at our disposal.

It has been extensively used by Todd (1927b), Hare (1934, 1935) and Spink and Keefer (1936), but the results obtained by these workers are not quite comparable. Both Todd and Hare took cognisance chiefly of the changes in the streptococcal population occurring after 1-3 hours' incubation, while Spink and Keefer observed the result after 24 hours' incubation. In our own work we have sometimes made counts in the early and late stages of incubation, at other times we have registered only the final result (24 hours).

Methods employed for determining the population changes occurring in serum

(a) A measured volume (5 or 10 c.mm.) of streptococcal culture suitably diluted was implanted into 200 c.mm. of fresh serum. After incubation in open or in closed tubes measured samples were withdrawn and explanted into melted blood agar.

(b) A 5 c.mm. volume of diluted culture was mixed with a 50 c.mm.

had usually resulted in abundant outgrowth of the cocci in the blood. Typical examples of the different effects observed with several strains are shown in table I. It seemed probable that the striking difference between the open and closed tubes might depend upon gaseous interchange through the cotton wool plug but it was not at all clear how this might affect the blood-streptococcus mixtures.

TABLE I

Effect on the survival of hæmolytic streptococci of agitation in human blood at 37° C

	Strains				
	Robb (mouse- virulent)	Turner V S (avirulent)	Richards (avirulent)	Richards (virulent)	Sapsford
No of cocci implanted	18,800	26,400	13,400	40,800	13,200
Surviving after 16 hours' agitation in closed tubes	α	α	8 millions	2.5 millions	α
Surviving after 16 hours' agitation in open tubes	0	0	30	0	0

The same sample of blood was used in the open and closed tubes with each strain.

The possibility that access of oxygen to the open tubes might favour phagocytosis was ruled out when we found that the streptococcal population showed similar changes if the blood had been heated to 45° C (to kill the leucocytes).

The conversion of glucose into lactic acid had also to be considered, but this change took place almost equally in open and closed tubes on incubation at 37° C (table II). We therefore investigated

TABLE II

Showing the progressive loss of glucose from blood during agitation at 37° C

Period of agitation of blood at 37° C (hours)	Sugar content of blood in open tubes (mg per cent)	Sugar content of blood in closed tubes
0	88	88
1	43	45
2	29	33
3	6	18

the more likely hypothesis that escape of CO₂ from the open tubes might adversely affect the metabolism of the streptococci. Gladstone, Fildes and Richardson (1935) had shown that "the

found that the pH of a sample of defibrinated blood after agitation in an open tube for 30 hours at 37° C had risen only to 7.95, as compared with 7.7 for a fresh sample examined at the same time (We are indebted to Dr Elford and Mr Jacobs of the National Institute for Medical Research for these tests)

It is very improbable therefore that the failure of streptococci to survive and grow in blood agitated in open tubes is due to pH changes, which are relatively small

Evidence that the loss of CO₂ from blood agitated in open tubes is chiefly responsible for the inability of the streptococci to survive and grow in such blood

Streptococci were incubated in blood with varying concentrations of CO₂, and their fate determined. These concentrations were regulated by three different methods, as follows

(1) *The "large atmosphere" method.* Heated blood (see footnote to p 445) plus streptococcus mixtures (total volume 15 c.c.) were agitated at the bottom of V-shaped tubes, the two limbs of which were connected by rubber tubing with the top and bottom respectively of a litre reservoir containing a known concentration of CO₂ in air. Since the V tubes were immersed in a water-bath at 37° C and the reservoir was at room temperature there was a continual circulation of the CO₂-enriched air through the V tubes. By varying the concentration of CO₂ in the reservoir it was possible to regulate the loss of CO₂ from the blood in the V tubes since this would proceed only until equilibrium with the air above it was established.*

In table IV it is seen that when the CO₂ content of the air space was kept above 0.7 vol per cent the streptococci were able to multiply freely. When the CO₂ content was below that figure they were unable to do so or died off completely.

(2) *The "small atmosphere" method.* In this the loss of CO₂ from the agitated blood was automatically regulated by varying the ratio of the volume of blood to that of the air above it in a closed tube (table V). Blood can liberate about half its volume of CO₂. Thus if 100 c.mm. are shaken in a 15×15 cm tube (volume approximately 15 c.cm.) 50 c.mm. of CO₂ will be liberated and the CO₂ in the air above the blood will rise to about 0.3 per cent.

* It will be evident that the CO₂ content of a fluid bears a fixed relation to the CO₂ content of the atmosphere above. The ratio depends on the temperature, and for blood at 37° C is about 0.5, that is to say whatever the CO₂ content of the air in the tube the fluid will contain half that concentration of free CO₂. The figures for total CO₂ content of blood used in this paper refer of course to bicarbonate plus free CO₂. The percentage of this total CO₂ which is free can be calculated by a physico-chemical equation if the pH of the blood is known. The more alkaline the blood, the smaller the proportion of CO₂ which is free. At pH 7.5 four per cent is free, at pH 8 about one per cent. When blood is agitated at 37° C in an open tube its alkalinity rises gradually and approximates to pH 8 when all the CO₂ is exhausted.

TABLE VI

Showing correlation between changes in the streptococcal population and the CO₂ content of the air in closed tubes (small atmosphere method)

Volume of infected blood	CO ₂ concentration in air of tube at end of experiment (vols per cent)	Number of streptococci per 100 c mm implanted in the blood (brackets) and after agitation in closed tubes for 24 hours at 37° C			
		Strain Fenner (type 6)	Strain Richards (type 3)	Strain Gillard (type 17)	Strain Sapsford (type undetermined)
100 c mm	0.27 (approx)	(5800) 30	(1500) 1,590	(1350) 150,000	(1580) 1,840
300 "	0.84 "	(5800) 1,200	(1500) 104,000	(1350) 5,500,000	(1580) 120,000
600 "	1.37 "	(5800) 28,000,000	(1500) 2,800,000	(1350) 6,400,000	(1580) ∞

Four other strains were tested in the same way. With all, we obtained a similar correlation between the population curve and the CO₂ content of the air of the tubes, but with two (strains Chapman and Murray) it was less striking than the examples shown in table VI, considerable growth occurring in 100 c mm of blood. Although these two latter strains therefore proved to be rather less sensitive to the diminished CO₂ concentration than the six others, they were quite unable to survive and multiply if the blood had been exhausted of its CO₂ (by shaking in an open tube for an hour or two) before it was implanted with streptococci.

(3) *The "regulated atmosphere" method.* The third way of adjusting the amount of CO₂ in the atmosphere was by using a fixed amount of blood in a closed tube, the blood having previously been shaken for a certain time in an "open" tube before the streptococci were implanted into it. By varying the period of preliminary shaking in the open tube the amount of CO₂ available for liberation into the air of the closed tube was regulated at will. The few experiments carried out by this method confirmed previous results.

Confirmatory evidence

The results obtained by these three methods strongly suggest that the loss of CO₂ from the blood agitated in open tubes is responsible for lack of growth of the streptococci. To make this still more certain it was necessary to show that blood made unfit for growth of streptococci by loss of CO₂ is able to support growth when CO₂ is returned to it. This is shown by the following experiment. Heated human blood was deprived of practically all its CO₂ by agitation in an open tube at 37° C and was implanted with streptococci and distributed by 300 c mm vols into 15 × 1.5 cm tubes. The air of these tubes was replaced by air containing known concentrations of CO₂, they were then closed with a rubber bung and agitated.

a part in this restriction of growth, but we have as yet not much evidence of this

Outgrowth of the streptococci from the beginning of incubation—i.e. without any primary phase of destruction or aggregation—has seldom been observed by us with human blood, but the strains we have used have not been cultivated repeatedly in human serum, unlike those of Todd (1927*b*), nor were most of them freshly isolated from very severe human infections as were those of Hare (1934), which showed immediate outgrowth in blood. It is of interest to note however that some of the strains we used were of high virulence for mice (after repeated passage). Even these strains usually showed a considerable reduction of the count during the first 2 or 3 hours of incubation with normal human blood.

Our experience of repeated observations on the blood of 11 normal individuals (laboratory personnel), embracing some 200 tests with 10 strains of hæmolytic streptococci (Griffith types 2, 3, 6, 11, 28 and four others unidentified) may be summarised as follows.

(1) The blood of all normal individuals is able to kill some strains and fails to kill others. We have frequently registered complete killing of streptococcal implants ranging from 1000 to 100,000 per c.c. of blood and occasionally even higher figures. Often, too, the test was not carried to the upper limit of killing. This is in conformity with the experience of Spink and Keefer (1936) and of Hare (1934).

(2) There is considerable variation between normal individuals in respect of a given strain (table VII), and we have observed variable effects with the blood of the same individual from time to time.

TABLE VII

Changes of streptococcal population in three normal bloods tested at the same time and under the same conditions

Blood of	Streptococcus, strain Robb, (erysipelas) type 3 mouse virulence high	
	Number of cocci implanted (brackets) and number surviving in 200 c.mm of blood after incubation in rotating tubes for 24 hours	
L C	(37) 1,200,000	(3700) 9,280,000
W R M	(37) 0	(3700) 0
R R	(37) 9,400,000	(3700) 13,860,000
	Streptococcus, strain Turner, (puerperal fever) mouse virulence low	
A T F	(248) 192,000	(24,800) \propto
W R M	(248) 1,500,000	(24,800) \propto
G H	(248) 0	(24,800) 2580

(3) The capacity of normal human blood to kill large numbers of hæmolytic streptococci was not related to recent infection by

RESULTS OBTAINED WITH HUMAN SERUM

Tillett (1937) reported a rapid falling off in the number of viable hæmolytic streptococci when certain strains were incubated with the serum of febrile patients. This falling off appeared to be a true bactericidal and bacteriostatic effect and not attributable to agglutination. His experiments were conducted in stationary wool-plugged tubes. The bactericidal effects did not occur when the tubes were incubated under anaerobic conditions. In a later paper Tillett and Stock (1937) expressed the view that the results obtained were referable to some bactericidal principle—the nature of which remains obscure—present in the serum of febrile patients and not in normal serum, but they recognised also that the increasing alkalinity of serum (above pH 9.0) occurring before and after implantation with streptococci exercised similar destructive action.

Our own observations deal with the fate of hæmolytic streptococci in the serum of normal individuals. In stationary tubes, open or closed, we found, as Tillett had done, that these streptococci were able to survive and multiply. Although they grew freely as a rule, the maximum reached was usually somewhat lower than that in defibrinated blood. Similar population changes occurred in closed tubes which were agitated during incubation, and this was so even if the serum used had previously lost some of its CO_2 by exhaustion *in vacuo* at room temperature.

If the serum was first agitated in an open tube at $37^\circ C$ for 20 hours or in a vacuum for half an hour at the same temperature and then inoculated, organisms were unable to multiply freely and were often killed rapidly during subsequent incubation (in a closed tube). During preliminary agitation at $37^\circ C$ in contact with air, or exhaustion *in vacuo*, there was a very considerable rise in pH —to 9.0 or even higher. Evidently there had been a considerable decomposition of sodium bicarbonate to carbonate $2NaHCO_3 \rightarrow Na_2CO_3 + H_2O + CO_2$. When a watery solution (0.2 per cent) of bicarbonate was agitated in an open tube at $37^\circ C$, 50 per cent of it was converted into carbonate after 20 hours *in vacuo* at $37^\circ C$ the change was much more rapid.

To determine whether the alkalinity of the serum would account for its bactericidal and bacteriostatic properties streptococci were planted into fresh human serum adjusted to different pH values, with subsequent agitation in closed tubes at $37^\circ C$. Control volumes of serum which had not been adjusted were similarly implanted with streptococci and incubated in closed and open tubes respectively. The pH levels reached after incubation and the changes in the streptococcal population are shown in table IX. A similar correlation between the streptococcal

fate of hæmolytic streptococci in their blood. Earlier work had brought to light the anomalous fact that the blood of mice and of rabbits under treatment by curative doses of sulphamylamide, although on chemical assay it contained at least as much of the drug as the blood of human patients similarly treated, had much less bacteriostatic and bactericidal effect upon streptococci implanted into it. A similar difference was also observed when the drug was added to the blood *in vitro*.

The observations we have made with regard to the fate of hæmolytic streptococci in the blood of rabbits and mice may be summarised as follows.

(a) In the blood of rabbits and mice, agitated in closed tubes, there was little or no initial reduction of the streptococcal count and even the smallest implants (1-20 cocci) always grew out freely within 20 hours. Apparently the leucocytes, unlike those of most samples of human blood, were unable to destroy even a very few streptococci, at least under the experimental conditions employed. Blood from 5 mice and 5 rabbits was used for these tests and 2 strains of group A streptococci (Griffith type 3 and one undetermined). Table X shows typical results.

TABLE X

Changes in streptococcal population in the unheated blood of mice and rabbits (closed tubes)

Blood	Strain Richards		Strain Turner	
	Number of cocci implanted (brackets) and number surviving in 200 c.mm of blood after incubation in rotating tubes for			
	2 hours	20 hours	2 hours	20 hours
Mouse A	(95) 4200	∞	(300) 150	∞
" B	(95) 5400	∞	(300) 480	∞
" C	(95) 6300	∞		
Rabbit D	(95) 2250	∞	(300) 5850	∞
" E	(95) 3750	∞	(300) 4800	∞
" F	(95) 4375	∞	(300) 4800	∞

(b) The blood of rabbits and mice provides a more favourable medium than that of man for the growth of hæmolytic streptococci. This was demonstrated in blood that was heated to 45° C for half-an-hour to eliminate the factor of leucocytic killing. A typical result is shown in table XI.

(c) The blood of rabbits and mice, when agitated in open tubes, frequently permits the growth of hæmolytic streptococci (5 strains out of 6 tested with the blood of rabbits, 2 out of 4 with the blood of mice) in spite of the fact that CO₂ is lost on agitation at 37° C as fast as or even a little faster than it is from human

It is difficult to say whether the *total* CO_2 values for the inoculated blood shown in this experiment represent a *free* CO_2 level as high as that required by most strains when growing in human blood, *i.e.* approximately 0.5 vol per cent. The *pH* of rabbit's blood after streptococci have grown freely in it in an open tube is 7.5. At this reaction and with a *total* CO_2 level of nearly 3 per cent we should expect the concentration of free CO_2 to be approximately 0.15 vol per cent, but, since some of the *total* CO_2 has been produced by the metabolic activity of the streptococcus, there is some doubt whether the usual relationship between CO_2 , bicarbonate and *pH* will hold good. If it does not, the level of available CO_2 in rabbit's blood during the growth of streptococcus may be equal to that required by that organism in human blood. It may be that the extra growth factor present in rabbit's blood (see below), by facilitating their rapid multiplication, enables the cocci to produce enough CO_2 for their needs before the supply in the blood gives out.

(2) The presence of special growth-promoting factors in animal blood enables vigorous metabolism of the streptococcus to take place in a medium of lower CO_2 concentration than it otherwise would.

The following observations lend support to this view and also provide evidence of the existence of a special growth-promoting factor in the blood of rabbits and mice.

(a) A 20 per cent addition of rabbit or mouse red corpuscles (washed and heated) to heated human blood enabled hæmolytic streptococci (strains Turner and Chapman) to grow out when the CO_2 concentration was too low to allow of growth without such an addition, *i.e.* in an agitated open tube.

It was possible to extract from the red corpuscles of the mouse a substance or substances which had the same effect as whole cells in facilitating the growth of streptococci in heated human blood. The hæmoglobin was precipitated by alcohol, the precipitate filtered off and the filtrate evaporated to dryness. The water-soluble fraction of this residue contained the growth-promoting factor or factors.

(b) The addition of one per cent peptone or Lemco, of mouse or rabbit urine or of alcoholic extracts of these urines, to heated human blood enabled streptococci to grow in open tubes just as animal red corpuscles had done. Human urine and lactose did not have this effect.

In connection with hypothesis (2) above, it is of interest to record also that some strains which grew very sparsely in a poor nutrient medium (trypsinised heart broth diluted eightfold) agitated in open tubes at 37°C gave four times as much growth in the same medium in closed tubes in an atmosphere containing 1 per cent of CO_2 . (With the Richards strain there was little difference.) Similarly in diluted mouse blood the strain Turner gave twice as much growth in an atmosphere containing 1 per cent of CO_2 as it did in an agitated open tube.

RESULTS OBTAINED WITH ANIMAL SERA

The changes in *pH* observed with the sera of rabbits and mice on agitation at 37°C in open and closed tubes respectively were

Similar results have been obtained on many occasions and leave no room for doubt that, if we wish to determine the maximum antibacterial effect of a given sample of blood, the rotating tube method is far better than the slide cell method. This statement is not necessarily applicable to bactericidal experiments with other bacteria or with the blood of animals.

Attention may be called to one practical point in connection with bactericidal tests. It has been observed by Dr E W Todd, Dr G Buttle and ourselves, and confirmed by Dr C B Dyson (1938), that hæmolytic streptococci (group A) in blood and serum are surprisingly sensitive to very small rises of temperature. Exposure to 40° C for a few hours was sufficient to kill the 5 strains we have tested. On one occasion the small amount of heat generated by an electric motor in the incubator led us to faulty conclusions.

DISCUSSION.

- (1) *What bearing have the data obtained with blood in vitro upon the initiation and development of human infections by hæmolytic streptococci?*

Two points may be made in this connection. (a) If it is legitimate to assume that the ability of human defibrinated blood to kill streptococci *in vitro* gives some indication of the capacity of whole blood to deal with such organisms in the circulatory system, and also perhaps a less clear indication of the defensive potentialities of the human fixed tissues, in which invading bacteria are usually first lodged, then it would seem to follow that certain individuals are much less susceptible than others to infection by hæmolytic streptococci, also, that by a bactericidal test of the blood, the degree of susceptibility of any person to a particular strain can be roughly estimated. (b) The CO₂ content of the circulating blood is at its lowest in the arterial blood leaving the lungs, where it has been in equilibrium with about 5 per cent of CO₂, and it will rise during the passage of the blood through the veins and capillaries. It is clear, therefore, that the CO₂ content of the circulating blood must at all times be greater than that in the blood agitated in the closed tubes of our experiments and therefore much more than enough to permit the survival and multiplication of any hæmolytic streptococci which reach the blood stream, *e.g.* from a septic thrombophlebitis or ulcerative endocardial lesion. The fact that, in spite of such favourable conditions for growth of streptococci in the blood stream, these microbes very seldom do multiply freely until the last few hours of life, strongly suggests that the cocci are continuously removed and destroyed, probably by leucocytes during the slow transit of the blood through capillaries and by the reticulo-endothelial system, and that this clearing mechanism is remarkably effective in human beings.

(3) *Why do hæmolytic streptococci (group A) need so high a concentration of CO₂ for their survival and growth in human blood?*

We cannot at present give an adequate explanation of this phenomenon. It has been suggested, in view of the fact that many bacteria need a small amount of CO₂ when growing in nutrient media, that this weak, easily diffusible acid is essential for the proper control of the acidity inside the bacterial cell. But the concentrations required for growth in human blood are very much higher than those required in animal blood or broth. It does not seem probable that so much more CO₂ should be needed for diffusion purposes in human blood than in the other media.

Since fat-soluble acids may also be able to diffuse through the bacterial envelope it was thought that growth might occur in the absence of much CO₂ if such acids were added to human blood.

A 0.1 per cent addition of the acids was made to human blood, which was then inoculated with cocci and agitated in open tubes at 37° C. The following fatty acids were used: heptanoic acid (CH₃ (CH₂)₅ COOH), undecanoic acid (CH₃ (CH₂)₉ COOH) and stearic acid (CH₃ (CH₂)₁₆ COOH). Even in the presence of these acids no growth occurred. With the following dibasic acids growth did occur: adipic acid ((CH₂)₄ (COOH)₂), sebacic acid ((CH₂)₈ (COOH)₂). When, however, solutions of adipic and sebacic acids neutralised to pH 7.4 were used no growth took place.

The results are conflicting, and the failure of the fatty acids to be effective makes the diffusion idea less probable.

A more probable explanation is that human blood lacks some factor necessary for the growth of hæmolytic streptococci, which carbon dioxide can replace. The fact that the addition of peptone, Lemco, mouse or rabbit blood or urine, or alcoholic extracts of the latter, enables hæmolytic streptococci to grow in human blood in the absence of much CO₂ indicates that the problem is primarily a nutritional one.

CONCLUSIONS

(1) The fate of hæmolytic streptococci in normal (defibrinated) human blood *in vitro* is a resultant of three factors, two of which (*a* and *c* *infra*) operate more or less effectively according to the set up of the experiment. These factors are —

(a) The efficiency of the destructive agencies of the blood (leucocytes, opsonic and pro-tryptic functions of the serum)

(b) The nutritive requirements originally present in the blood

(c) Chemical changes occurring in the blood during incubation. The most important of these is the rapid loss of CO₂ which occurs if the blood is agitated in an open tube at 37° C. When blood is agitated in a closed tube with small air space, the CO₂ loss is not

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In previous experiments (Evans and Maitland, 1939) it was shown that with living suspensions the titre was twofold and sometimes fourfold the titre shown with heated suspensions.

The sera were also tested by slide agglutination. A loopful of living suspension (10^{11} organisms per c.c.) made as described above was mixed with a loopful of undiluted serum. The tests were read under the microscope. The positive reactions were very definite and almost immediate, marked clumping occurring throughout the drop. The control suspensions and negative reactions showed no granularity.

Test for neutralisation of toxin

Pertussis toxin in the form of an extract of frozen and thawed and ground bacilli was freshly prepared as described previously (Evans and Maitland, 1937). Twofold dilutions from 1:40 to 1:1280 were made in water. To 0.15 c.c. of each dilution 0.15 c.c. of undiluted serum was added and the mixture left at room temperature for $\frac{3}{4}$ -1 hour. Intradermal inoculation was then made of 0.2 c.c. of each mixture into the shaved back of a rabbit. Three whooping cough sera and one normal adult serum as a control were tested on one rabbit. The same control serum was used throughout. The size of the lesions was measured after 2, 4 and 6 days. The end-point was taken as the highest dilution of toxin which produced a necrotic lesion.

Results

The results of complement fixation, agglutination and neutralisation tests are shown in the table. With the exception of serum 1a (taken 4 days after onset of illness) all fixed complement and all but one agglutinated. None however neutralised toxin. In any one rabbit the size, rate of development and appearance of the lesions as well as the end-point were identical for all four sera. In preliminary tests no difference was observed between toxin mixed with control serum and toxin alone. It is seen from the table that some variation in reactivity between individual rabbits occurred which necessitated a control for each animal. Sera 1a, 1b and 1c came from the same patient, the first sample (after 4 days' illness) had no antibodies, later samples fixed complement and contained agglutinin, but none neutralised toxin.

Discussion

These results confirm our earlier experience and support the view that *pertussis* toxin is not antigenic. The toxin is very labile, which adds to the difficulty of separating it from other material in the extract and determining its chemical nature. At present it is not possible to explain by experimentally obtained data why the toxin should not be antigenic. In view of its lability the possibility has been considered that neutralisation might occur *in vivo* by some mechanism other than direct combination of toxin and antitoxin. On the other hand rabbits and guinea-pigs which were immunised by living *H. pertussis* reacted normally to the intradermal injection of toxin (Evans and Maitland, 1937). We have tried to detect neutralisation by injecting 0.1 c.c. of convalescent serum intradermally into the shaved back of a rabbit and one hour later injecting 0.1 c.c. of toxin into the same area. The preliminary injection of serum did not neutralise the necrotic effect of the toxin.

Summary

Twenty-three sera from cases of whooping cough failed to neutralise *pertussis* toxin.

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AGGLUTINATION AS A DIAGNOSTIC TEST FOR
WHOOPING COUGH

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Complement fixation is recognised as a test of value for confirming the diagnosis of whooping cough. Agglutination has been tried for the same purpose, but the literature on this subject suggests that agglutination is too inconstant in whooping-cough sera and occurs too often in normal sera to afford a reliable test (Bordet and Gengou, 1907, Wollstein, 1909, Winholt, 1915, Povitzky and Worth, 1916, Donald, 1938). We have compared the relative diagnostic value of these two tests. The technique is described in the preceding paper. The results may be considered in three groups.

(a) *Cases of whooping cough diagnosed clinically whose sera fixed complement* The 21 cases in this group, none of whom had had vaccine, are shown in the table of the preceding paper (p. 467). Sera 1a, 1b and 1c came from the same case and as serum 1a was taken on the fourth day after onset and did not fix complement it is excluded. Of these 22 sera 18 showed slide agglutination and had a tube agglutination titre of 1.40 or higher (table). Three showed slide agglutination only. Thus with one exception (no. 18) sera which fixed complement also agglutinated.

(b) *Cases or suspected cases of whooping cough whose sera did not fix complement* In this group there were 15 cases (table). Cough plate

TABLE

*Comparison of agglutination and complement fixation
as diagnostic tests for whooping cough*

	Number of sera	Number positive by		
		slide agglutination	tube agglutination	complement fixation.
(a) Cases of whooping cough whose sera fixed complement	22	21	18	22
(b) Cases or suspected cases whose sera did not fix complement	15	4	4	0
(c) Controls (cases without clinical signs of whooping cough whose sera did not fix complement)	39	4	3	0
Total	76	29	25	22

examinations were not made. None of the cases had vaccine. Of the 15 sera 4 agglutinated, the respective titres being 1/80, 1/80, 1/320 and 1/640, and these sera also showed slide agglutination. The remaining 11 sera did not agglutinate by either method. It is probable that the agglutinin in the serum of the four exceptional cases indicated present infection, the ages of these patients were between 15 months and 3 years.

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616 . 832 . 9—002—022 : 576 . 851 . 4 (*Bact monocytogenes*)

A CASE OF MENINGITIS DUE TO *BACTERIUM*
MONOCYTOGENES

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(PLATE LIV)

As there are still comparatively few published records of disease in man caused by *Bact monocytogenes*, it is desirable that new cases should be reported. References to previously published cases in man and animals may be found in the papers of Webb and Barber (1937) and Barber (1939).

Case record

The patient, a boy aged 17 months, from Grangemouth, Stirlingshire, was healthy until, in January 1938, he developed chickenpox. A week later signs of meningitis appeared and he died on the seventh day thereafter, three days after admission to the Royal Edinburgh Hospital for Sick Children.

Lumbar puncture was performed three times while he was in hospital. On each occasion the cerebro-spinal fluid was under pressure and turbid, and microscopical examination revealed large numbers of mononuclear and polymorphonuclear cells and intra- and extracellular fine Gram-positive rods (fig 1). These showed a tendency to chain formation, but were most often single and lying at an angle to one another. Long, short and sometimes curved forms were present. No other organisms were seen. Aerobic culture on blood agar produced a pure and fairly profuse growth from each specimen in twenty-four hours. The results of differential cell counts on two specimens of cerebro-spinal fluid are set out in the table.

Both show a considerable content of large mononuclear cells, amounting, in the case of the specimen withdrawn on the day of death, to no less than half the total number of cells present. Unfortunately no blood count was done.

At necropsy a typical suppurative lepto-meningitis was found. The purulent exudate was thickest over the vertex and frontal lobes, but was present at the base also. Both middle ears contained pus, though there had been no clinical signs of otitis media. Nothing else of interest was found in any of the organs except the liver, which was pale and showed, through the peritoneum, numerous grey flecks about 1 mm in diameter. On section some of these were seen to extend a short distance down from

1.

and sometimes in the spleen and elsewhere. Intravenous injection of similar doses usually killed the animal rapidly, before any lesions in the liver could be detected with the naked eye. The organism was recoverable from the blood, liver, spleen and kidneys. Both macroscopically and microscopically the appearance of the liver lesions resembled very closely the published descriptions of Webb and Barber. Numerous small areas of focal necrosis were present, with large numbers of bacteria in all the foci. Their distribution had no relation to the zones of the hepatic lobules. Dense infiltration with polymorphs and mononuclear cells occurred around the foci (fig 3). The lesions found in other abdominal organs were similar. No intracranial lesions occurred.

Commentary

From these pathological and bacteriological findings, as well as from the clinical history, there is no doubt that this was a case of meningitis due to infection with *Bact monocytogenes*. As in previously recorded cases, no clue to the source of the infection was discovered. Otitis media was found at necropsy, and it may be significant that this condition was present in two of the cases described by Burn (1936). There was, however, no bacteriological evidence in our case that the organism was present in the middle ears. It is generally suspected that *Bact monocytogenes* may be more widely distributed among animals than is at present realised. Efforts were made to trace any contact with healthy or diseased animals in this case, but without success.

We are indebted to Dr Lewis Thatcher, under whose care the patient was admitted to hospital, for permission to publish this case, to Professor Robert A Webb, Royal Free Hospital, London, for serum and for his interest in the bacteriological investigation, to Professor T J Mackie for his interest and advice, and to Miss Joyce Cranfield, B Sc, for assistance with the animal experiments. The cost of experimental animals was defrayed out of a grant from the Earl of Moray Endowment of Edinburgh University.

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STENOSIS OF THE PULMONARY VALVE DUE TO SEVERE ATHEROMA

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(PLATE LV)

Although atheroma of the pulmonary artery and its branches is not uncommon, severe involvement of the pulmonary valve must be a rarity since extended search of the literature fails to disclose a case. In this

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A SOLITARY CYSTIC METASTASIS IN THE BRAIN FROM A CARCINOMA OF THE BREAST

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(PLATE LVI)

Pronounced cystic change in metastatic carcinoma in the brain is unusual, but is occasionally of such a degree that the cancerous epithelium is present only as a microscopically thin layer lining a large cavity. I have already described an example of this kind and have referred to other records of cystic metastatic growths in the brain (Willis, 1934, p 355 and fig 89). Carcinoma of the lung is the most frequent source of such metastases. The following case is noteworthy in that the only metastasis from a mammary carcinoma was a large cyst lined by cancerous epithelium situated in the cerebrum.

Case report

M H, a woman 64 years old, was admitted to hospital in November 1937. In 1931 she had received radium treatment for a growth of the left breast. Ever since then she had had a deep ulcer at the site and of recent months the ulcer had extended and had begun to bleed. Examination showed a large foul cavity in the left mammary region, exposing and partly destroying several ribs. The patient died a few days after admission.

Post-mortem examination. The cause of death was radium necrosis of the chest wall, with extension into the lung and pneumonia. There was no sign of any residual growth in the tissues around the necrotic area, and careful examination failed to reveal any metastatic deposits in any of the thoracic or abdominal viscera or in the skull, ribs or vertebrae. In the white matter of the occipital lobe of the right cerebral hemisphere, separate from the ventricle, there was an irregularly rounded cavity 3 cm in diameter (fig 1) with brownish watery contents. Most of the inner surface of its wall was smooth or only finely nodular, but at one part there was a firm projecting nodule 6 mm in diameter in which some small foci of calcification were felt on cutting across it. This area was the only part of the lesion which to the naked eye suggested the presence of tumour tissue.

Microscopically, most of the cavity in the brain was lined by a thin layer of cancerous epithelium, which in many places consisted of a single layer of cubical cells (fig 2), while in other places it was irregularly stratified. The nodule of firm tissue consisted of disorderly spheroidal-celled and adenocarcinoma with areas of necrosis and calcification (fig 3).

Comments

The following features of the case are noteworthy.

(a) *The cystic character of the metastasis.* Except for the small nodule, to the naked eye the lesion suggested some kind of simple cyst in the brain.

(b) *The solitary nature of the metastasis.* Thorough examination of the remainder of the brain and of all other parts of the body failed to disclose any other metastatic growths. The condition is thus an interesting instance

examined For each European case an accurate history of service in the local gold-mining industry was available, but not for previous occupation elsewhere Such industrial records were unfortunately not available for the native miners

In the digestion of lung tissue trypsin was replaced by papain powder, which does not require alkali and which proved a more powerful reagent Small samples of lung tissue were minced and shaken with 10 c.c. of water and a generous pinch of papainum After two or three days at 54° C., when the tissue was completely reduced to a smooth slime, the suspension was filtered through gauze, and the filtrate centrifuged Films from the sediment were dried and mounted in Canada balsam

"Curious bodies" were found in 19 (59 per cent) of the 32 cases—8 Europeans and 11 natives In 2 natives, both showing frank asbestosis, myriads of pale-cored bodies were present In all the other positive cases the bodies were few in number One European and 3 natives showed pale-cored bodies only, 3 Europeans and 4 natives showed black-cored bodies only, and 4 Europeans and 2 natives showed both types in about equal numbers

Of the 19 positive cases, gross examination of the lungs showed typical lesions of asbestosis in 2, minimal silicotic fibrosis in 12, and no evident lesions in 5 Of the 13 negative cases, 5 showed minimal silicosis similar to that seen in the 12 positive cases Histological examination was not carried out

In none of the positive cases was there any history of work in the asbestos industry, though such occupation may be assumed for the 2 natives showing typical asbestosis In the other 17 cases the presence of the bodies had no constant relation to the presence of fibrotic or other lesions Since my 1934 investigations I have found black-cored bodies frequently in the lungs of coal-miners and trimmers, and I am convinced that they are not closely related to the development of anthraco-silicosis The "curious bodies" presumably arise from the chance inhalation of mineral fibres or spicules, but it is not yet possible to say with certainty whether they are produced only by silica-containing minerals It is of interest, however, that whereas typical asbestos bodies can regularly be produced by the experimental inhalation of asbestos dust, the black-cored bodies have not so far been reported as the result of dusting experiments

In the present series, apart from the 2 asbestosis cases, 10 cases, 5 Europeans and 5 natives, with no history of asbestos work, showed typical pale-cored bodies, the origin of which is a matter for speculation There is, however, in the Rand, a narrow layer of banket of a fibrous nature, the silicious fibres of which might be responsible

Summary

"Curious bodies" of two morphological types have been observed in the lungs of South African gold-miners with no history of exposure to either asbestos or coal

There was no evident relationship between their occurrence and the presence of lung lesions

The findings suggest that these "bodies" may arise from the chance inhalation of mineral fibres not necessarily related to asbestos or to coal

This investigation was carried out during the tenure of a Dorothy Temple Cross Fellowship I am indebted to the Director of the South African Institute for Medical Research for permission to publish this work, and to the Medical Staff of the Pathology Department there for allowing access to material My thanks are also due to Professor W. H. Tytler of the Welsh National School of Medicine for help in the presentation of this article

with ammonium sulphate and the native globin precipitated by complete saturation with ammonium sulphate. The protein was dissolved in a small volume of water, dialysed until free from ammonium sulphate and dried *in vacuo* over phosphorus pentoxide. The identity of the protein was confirmed by spectroscopic examination of the product of its combination with hæmatin. Hæmatin solution, prepared by the method of Anson and Mirsky (1929-30), was added to a solution of globin and the mixture treated with sodium hydrosulphite. The spectrum of reduced hæmoglobin was observed, which on treatment with carbon monoxide was changed to that of CO-hæmoglobin. Denatured globin under this treatment would give the spectrum of a hæmochromogen, unchanged by the action of carbon monoxide.

Experimental

Five strains of recently isolated *Staphylococcus aureus* were used. Suspensions of about 500 million per c c were made in saline by washing off the growth from agar slope cultures incubated for 24 hours. One c c quantities of these suspensions were pipetted into a series of tubes, centrifuged, and the supernatant fluid removed. One c c of each of the globin solutions to be tested was then added to the bacterial deposit, the whole shaken up and incubated at 37° C for 3 hours. In some experiments the mixtures were allowed to stand at room temperature for 3 hours but it was usually found that better results were obtained at 37° C. Marked agglutination of the staphylococci occurred. The tubes were then centrifuged, the supernatant fluid pipetted off, the deposit washed twice with normal saline and resuspended in normal saline. A drop of a leucocytic cream, prepared by centrifuging citrated guinea-pig's blood and washing the leucocytes four times with saline, was added to a few drops of the bacterial suspension and the whole incubated for 90 minutes at 37° C. Films were stained by Leishman's method. Control tubes of bacteria treated with saline and tubes containing bacteria and normal guinea-pig serum were set up to compare the effect of the artificial opsonin and the serum.

Concentrations of 0.5, 0.25 and 0.1 per cent of globin in distilled water gave distinct agglutination of the staphylococci, the effect being more marked with the higher concentrations. With all these concentrations of globin opsonisation occurred, so that on the addition of leucocytes there was very obvious phagocytosis, but less than when normal serum was the opsonising agent.

In all our experiments with artificial opsonins we have noted a variability in their action from one strain of organism to another, and even from one experiment to another using the same strain. We suggest that chemical differences in the bacterial surfaces are responsible for the variations in results. Ferric salts, which are the strongest of the inorganic artificial opsonins, were generally consistent in their action and globin is the most consistent of the organic artificial opsonins which we have tried, although it is more variable than ferric salts.

It was deemed of interest to ascertain if hæmoglobin itself had a similar opsonic action to that of globin. For this purpose defibrinated ox blood and rabbit blood were employed. The blood was centrifuged, the supernatant fluid removed, and the packed red cells washed several times with normal saline. To quantities of 0.1, 0.25, 0.5 and 1.0 c c of red cells were added 5 c c of distilled water, the whole being mixed thoroughly together and left on the bench for 30 minutes to allow lysis to occur. The tubes were centrifuged and 1 c c amounts of the supernatant fluid added to staphylococci, following the procedure described above for globin. No opsonisation of the staphylococci was observed when using hæmoglobin prepared in

him with a son of 17 and a daughter of 10. To them we would offer our sincerest sympathy

Shaw had been a member of the Society since 1929 but had had little opportunity of attending our meetings

J S Y

Emmeline Wade.

1898-1938.

DR EMMELINE WADE, who died on 3rd December 1938 at the age of 40, was at the time of her death assistant bacteriologist in the Public Health Department of the University of Durham Medical School, King's College, Newcastle-upon-Tyne. She had been a member of the Society since 1931

Dr Wade was a daughter of Mr Thomas Wade, a schoolmaster in Oldham. She was educated at Queen Elizabeth's Grammar School, Middleton, Lancs., and Manchester University, where she graduated in Medicine in 1922. After a number of clinical appointments, including that of assistant school medical officer at Middlesbrough, Dr Wade took the Diploma in Bacteriology of Manchester University and subsequently, in 1929, joined the staff of the routine section of the Bacteriological Department. In 1932 she was appointed to a lectureship in pathology in the University of Liverpool, where she was engaged largely upon bacteriological work and the clinical pathology of the Liverpool Maternity Hospital. In the six years she was in Liverpool Dr Wade quietly and efficiently filled an important niche in the Pathological Department. She was responsible for a large part of the bacteriological work and taught regularly and effectively in the practical classes. Gradually, however, she came under the shadow of the illness which was to terminate her life. In earlier years she had suffered from scarlet fever and had developed a chronic nephritis, the symptoms of which now became more and more obtrusive. Nevertheless she went on with her work quietly and courageously, refusing to bow before the inexorable progress of the malady. In the summer of 1937 came a breakdown which was followed by resignation of her lectureship. However, matters improved as a result of a long rest and in the spring of the following year she felt fit to resume work and went to the post at Newcastle.

Here for eight months she so ordered her life as to ensure that, despite the precarious state of her health, she could take a full share in the routine work of the laboratory—work which appealed to her, which seemingly afforded her a heartening interest in life, and for which her qualifications were of the highest order. She

BOOKS RECEIVED.

Bijdrage tot de kennis der typen van het *Corynebacterium diphtheriae*

By B W L SIEMENS Assen van Gorcum & Comp N.V. 1938
Pp 113, 21 figs Dutch florins 2 90

This thesis, presented to the Medical Faculty of the University of Amsterdam, takes the form of an extensive monograph on the type differentiation amongst diphtheria bacilli first suggested in Leeds. Besides presenting a more comprehensive survey of the literature of the subject than any yet published, the author records some limited observations on the distribution of the diphtheria types in Amsterdam, Rotterdam and Groningen.

Place	No of cases and carriers examined	Gravis	Inter-medius	Mitis
Amsterdam	90	17	0	73
Rotterdam	56	50	0	6
Groningen	5	0	3	2

It is interesting to note in this connection that from 1927 onwards the yearly mortality from diphtheria has been from three to eight times greater in Rotterdam than in Amsterdam. The small number of strains obtained from Groningen was due to the fact that diphtheria there was sporadic and slight. The author's chief contribution, however, is a careful appraisal of the significance of the suggested type differentiation, based on a purely bacteriological study of phenomena of variation within the types and of the existence and characters of atypical forms. The background of these studies is provided by van Loghem's application of genetic theory to the study of bacterial races.

Two points in technique are specially stressed, these are the value of Murray's illuminator (this *Journal*, 1935, xlv 97) and the importance of "super-isolation"—requiring a margin of 1.5 cm of medium free from growth in the vicinity of the colony to be examined—for the determination of the type colony characters. By adopting these methods, it is possible to classify a number of atypical strains with one or other of the three types first described.

It is not possible in a review to follow in detail the author's experimental work and argument but his main conclusions may be stated. Under the term "phænotypus" should be included all the various forms which a race or "genotypus" may exhibit under various external conditions, some of these variants or adaptation forms may retain their changed form with considerable tenacity. A summary of all the published work on this subject leads him to give most attention to the variants described by Christison and by Robinson. These are carefully considered in the light of his own work, including the introduction of cultures in collodion sacs into the peritoneum of guinea-pigs which have received injections

become the illusion of to-day, to-day's idea may become the illusion of to-morrow 'For', says Meredith, 'the mastery of an event lasteth among men the space of one cycle of years, and after that a fresh illusion springeth to befool mankind' Doubtless many masters of the event will follow after Darwin and Bateson in wielding the sword of Aklis, and through the dispelling of illusion after illusion mankind may eventually encounter the ultimate residue, perhaps the ultimate of all illusions, which we optimistically designate as truth "

Climate and acclimatization

By SIR ALDO CASTELLANI Second edition London John Bale, Sons & Curnow 1938 Pp x and 198, 12 text figs and 4 plates 10s

In his preface the author says that he does not aim at being exhaustive and that his little book is merely a collection of notes on some features of the problems of climate and acclimatisation The disclaimer is justified for, apart from a few paragraphs on the cause of mountain sickness and on the effect of extreme cold, the subject-matter of the book is confined to the influence of tropical climates on Europeans and the adjustment of man to environments in which, either from high temperature or moisture or a combination of the two, the dissipation of animal heat is impeded

The rest of the prefatory statement is unduly depreciative Parts of the book do, indeed, resemble a scientific scrap-book but where the author gets away from his notebooks he is vigorous and clear The author is a learned physician who has resided for many years in the tropics and has had experience of campaigning in tropical climes He is, moreover, widely conversant with scientific literature For these reasons his observations and reflections have a peculiar value, even if their presentation be rather chaotic

Plenteous use is made of extracts from scientific and medical journals though generally quite uncritically The reader is left to imagine that their length is a measure of the importance that the author attaches to the views and conclusions expressed in them However, the text is well supplied with references to original sources (over 200), which the serious student may consult and the chaos is mitigated by a good index

The first chapter is mostly devoted to an elementary statement of the geo-physical causes for different climates Chapter 2 opens with a useful resumé of the physiology of thermal adjustment The effects of high temperature and humidity on the temperature of the body and on various bodily systems are then considered and it ends with an account of the aetiology, pathology and treatment of heat stroke and the minor forms of heat trauma Chapter 3 is, unexpectedly, devoted to meteorology but contains also observations on the biological action of light of different refrangibility and on the causes of sunburn and pigmentation In chapter 4 the question of acclimatisation is discussed Observations are cited which show that the mortality of Europeans in the tropics need not exceed that of temperate climes, provided the liability to malaria and other diseases which, for various reasons, are peculiar to or prevalent in the warmer parts of the world, can be minimised by sanitary measures The possible fallacy of this conclusion from the existence of a selected population of Europeans with a very different age distribution to that in their mother countries is pointed out The author emphatically disagrees with those enthusiastic sanitarians who believe that a tropical climate itself is not prejudicial He is convinced

that dealing with the authors' own work, although, in view of the valuable work of Griffith and Allison in this country, exception must be taken to the statement that agglutination has not become of any practical value in diagnosis or in the identification of scarlet fever streptococci. The reader will also look in vain for information as to the Lancefield method of grouping hæmolytic streptococci by a precipitin test. The bibliography is extensive but its perusal confirms the impression that the authors have ignored the recent important contributions of British workers to this subject.

Iodine metabolism and thyroid function

By A W ELMER London Oxford University Press, Humphrey Milford 1938 Pp xvii and 605, 9 figs. on 6 plates and 14 text figs 30s

Those who are interested in the study of the thyroid gland must perforce concern themselves with the metabolism of iodine, an attempt such as is represented by Dr Elmer's book to collect and review the widely scattered information on the subject is therefore to be welcomed.

Owing to the minuteness of the amounts of iodine involved in bodily processes and to the low concentrations in which the element occurs in nature, the study of the metabolism of iodine is in essence a micro-chemical problem, it is fitting therefore that the brief historical introduction to Dr Elmer's book should be followed by a comprehensive review of methods of iodine determination. It is helpful also to have a clear indication, based on personal experience, of the author's own preferences among the numerous methods which have been proposed.

The second and largest major section of the book comprises an exhaustive treatment of the amounts and nature of the iodine compounds found in the normal organism and of physiological factors which may affect the metabolism of iodine, whilst the final section deals with iodine metabolism in pathological conditions, mainly of course those of the thyroid.

The physiological section contains a good discussion of the mode of combination of iodine in the thyroid and of the nature of the thyroid secretion, whilst considerable interest also attaches to the author's remarks, in the final portion of the book, on thyrotoxicosis. Neither of these sections, however, is so satisfactory as the chemical review, since the author does not succeed in making it easy, even for a reader with some knowledge of the original literature, to select the well established facts from the lamentably large number of insecurely based observations.

It is unfortunate that more trouble has not been taken with the production of the book, the frequent misspellings and errors in phraseology have been a source of considerable irritation to one reader at least. Despite these deficiencies, however, Dr Elmer's monograph stands alone in its field and will undoubtedly be found of great use by many workers in biological and medical science.

C R H

Methods of tissue culture

By RAYMOND C. PARKER London Hamish Hamilton Medical Books 1938 Pp xxxii and 292, 63 text figs 24s

Methods of tissue culture is a valuable addition to the books already published on this subject. It sets out to be an account of the methods

proficiency have been combined with engineering skill in the production of a workable method for the investigation of animal organs for extended periods of time under artificial conditions, by which is now realised a dream of generations of physiologists, pathologists and biochemists

From the purely biological point of view the value of the book lies rather in pointing the way to future investigation than in what has so far been achieved. The method has been established, many fields have been explored, but few if any have been completely mapped. In the early stages of a technique, feelers must be put out in many directions, but if a criticism can be levelled at all, it is that too many feelers have been put out and none has probed sufficiently thoroughly. It is left for others to follow the extremely precise instructions and accounts of the technique given herein and to build up the solid structure of knowledge concerning the behaviour of organs under experimental conditions, a structure whose foundations are now firmly laid and for which the plans are here sketched out.

The particular media best suited to culture of different organs have not yet been satisfactorily determined, but the opportunity now presents itself for the construction of a purely synthetic medium in which the function and properties of every constituent are known. The media described as having been used successfully up to the present time seem to be somewhat empirical. The urgent problem of the perfusion of the kidney has been attempted, but, with the usual perversity of that organ, it has proved difficult and is still far from solved. The addition of blood corpuscles to the perfusion fluid is found to help, but why?

The method appears to have two main difficulties. Firstly, it can only be satisfactorily carried out by a team of workers. Secondly, it is difficult to establish adequate control experiments. In the account given of the changes in the thyroid gland under different conditions of culture it is difficult, in spite of the excellent photographs, to decide how much is natural variation and how much is due to the experimental conditions.

Although somewhat grandiose in places, and in one or two instances loose and inaccurate, the book as a whole is clearly written, beautifully illustrated, well produced and very stimulating.

The adrenal cortex and intersexuality

By L. R. BROSTER, CLIFFORD ALLEN, H. W. C. VINES, JOCELYN PATTERSON, ALAN W. GREENWOOD, G. F. MARRIAN and G. C. BUTLER
London: Chapman & Hall, 1938. Pp. xii and 245, 26 figs on 23 plates and 7 text figs. 15s.

This book is "dedicated to the humanity and generosity of Viscount Wakefield of Hythe", whose support of the researches on which it is based is further acknowledged in Sir Walter Langdon-Brown's foreword. The subject matter is divided into four sections, of which the first (clinical and surgical) is by L. R. Broster, the second (psychological) by Clifford Allen and the third (pathological) by H. W. C. Vines. The fourth section is biochemical and consists of two chapters, one by Jocelyn Patterson and Alan W. Greenwood, the other by G. F. Marrian and G. C. Butler.

This monograph is an admirable example of what can be achieved by the combined clinical and laboratory study, by a team of workers, of a very complex medical problem. Broster and Vines have already written on the subject of the adreno-genital syndrome and adrenal

Chemotherapie Ergebnisse, Probleme und Arbeitsmethoden

By Dr MANFRED OESTERLIN Brunswick · F Vieweg & Son 1939
Pp viii and 359, 39 text figs R M 22 5 (bound), 20 (unbound)

Recent Advances in chemotherapy

By G M FINDLAY Second edition London J & A Churchill, Ltd
1939 Pp x and 523 21s

The titles of these books indicate that neither gives a full account of the subject of chemotherapy Oesterlin's work is divided into two sections The first or biological part deals—often very briefly—with infections due to trypanosomes, spirochaetes, plasmodia and halteridia, amœbæ, piroplasms and helminths, especially in so far as they are test objects for chemotherapeutic investigation The second or chemical part is concerned with the drugs which have been used in the various infections In this the author frequently develops his own views on the relations between chemical constitution and biological action His personal observations in various branches of chemotherapy make the exposition very suggestive The insistence that the physical or physico-chemical properties of the drugs often have a decisive influence on chemotherapeutic action is highly pertinent

Findlay's work is the second edition of that originally published in 1930 It includes extensive sections on the chemotherapy of bacterial and virus infections The author suggests that his book should be regarded as a supplement to Fischl and Schlossberger's *Handbuch* It fulfils this object in the sense that the series of drugs dealt with is brought up to date very fully Unfortunately, however, there is no publication with adequate detail which gives a general exposition of chemotherapeutic investigations Accordingly the reader who takes up either of the books under review is in much the same position as one would be if presented with similar accounts of recent work in pathology or bacteriology, supposing that no modern systematic text-books on these subjects were available So both Findlay's and Oesterlin's monographs are likely to be read with most interest and profit by specialists who have themselves worked on some aspect of chemotherapy, while they may prove bewildering to others who seek a sound groundwork in a rapidly extending department of knowledge

C H B

Statistical methods for research workers.

By R A FISHER Seventh edition Edinburgh Oliver and Boyd
1938 Pp xv and 356, 12 text figs 15s

When a book has reached its seventh edition within a space of fourteen years there is little room for doubt that it is fulfilling the function for which it was designed and is being of real assistance to research workers in the statistical treatment of their data Extensions and improvements dealing with the newer developments of the subject have appeared in successive editions of Professor Fisher's book In the present issue there is given a fuller introduction to the theory of orthogonal polynomials than in the last, and another section has been added to give an outline of the important new subject of the use of multiple measurements to form the best discriminant functions of which they are capable

and in 1936 a fourth. In each instance death had ensued within four days of severe burns and the liver presented a remarkably uniform and distinctive picture.

All were coroners' cases involving healthy individuals who were accidentally burned while at work. Each case was immediately taken to hospital* and treated in a similar manner with tannic acid spray. Each succumbed to toxæmia and came to autopsy within a few hours of death.

Clinical data

Case 1 A 39 32 Female, æt 51. Clothes were ignited by burning wax. Approximately one-third of body surface burned to 2nd and 3rd degree. Admitted to hospital 2 hours later in a state of shock and unconscious. The burns were effectively tanned in 24 hours. In spite of transfusion she remained unconscious and died in 48 hours.

Case 2 A 157 34 Male, æt 37, was sprayed with burning petrol and sustained 2nd and 3rd degree burns over one-third to one-half of body surface. Condition favourable for 24 hours, during which time tannic acid treatment applied, then temperature rose to 102° F, where it remained till death from toxæmia on 4th day. Intravenous glucose administered during illness.

Case 3 A 306 34 Male, æt 55, was scalded by steam from an exploded boiler, sustaining 2nd and 3rd degree burns over one-half to two-thirds of body surface. Death after 2 days of unconsciousness. Tannic acid treatment.

Case 4 R 145 36 Male, æt 21, was scalded by steam while at work. Approximately one-half of body surface affected by 2nd and 3rd degree burns. Admitted to hospital within one hour in profound condition of shock from which he did not rally up to time of death from toxæmia on 4th day. Tannic acid treatment and intravenous glucose.

Post-mortem findings

The post-mortem findings in the main were parallel in all four cases. There were the marks of severe toxæmia: acute parenchymatous degeneration of myocardium, liver and kidneys, a reacting, mushy spleen, weeping hæmorrhages of mucous membranes, particularly in stomach and renal pelves, and hæmorrhages into the lungs, accompanied in three cases by thrombosis of finer branches of the pulmonary arteries. Case 2 was slightly jaundiced at death and the stomach showed acute ulceration. Case 4 had about 500 c.c. of clear ascitic fluid in the peritoneal cavity.

The liver changes, similar in each case, engaged our particular attention. In the gross there was slight enlargement due to cloudy swelling. The weight varied from 1360 to 1720 g. The parenchyma was pale and possessed a yellowish or ochre tint resembling the boxwood colour said to be characteristic of the yellow fever liver.

* Three cases were treated at the Toronto General Hospital and the autopsies were performed in the Department of Pathology, University of Toronto.

The inclusion phenomenon was confined to parenchymal cells and was not observed in tissues other than the liver

Discussion

We do not know if all cases of fatal burns present the liver changes described above, but we may take it as evidence to the contrary that such changes have apparently not been recorded previously (Vogt, 1929). Here, however, we have four cases with a highly distinctive liver picture which seems on the face of it to be linked with the toxæmia of burns.

We do not know precisely what it is that poisons the victim of burns. Some toxic substance capable of profoundly harmful effects is undoubtedly elaborated in the damaged tissue, but its identity has not been established. Wells (1925) gives a good account of the chief contributions on the subject up to that date. A more recent review by Rosenthal (1937) suggests a histamine-like substance as the toxic agent.

The significance of nuclear inclusion bodies is exhaustively treated by Cowdry (p. 533), who says "one naturally looks for a virus when such inclusions" (*i.e.* type A) "are observed. Often a virus has been discovered, but there are exceptions." This seems to summarise the present state of our knowledge. Olitsky and Harford (1937) produced the inclusion phenomenon experimentally by the injection of certain aluminium and ferric compounds. VonGlabn and Pappenheimer (1925), Farber and Wolbach (1932), Covell (1932), Russell (1932), and Pappenheimer and Maechling (1934) have all described visceral inclusions without evidence of a concomitant virus. To our knowledge, however, inclusions so numerous and typical as in these cases of burns have never been observed before except in virus diseases. Klotz always leaned to the view that nuclear inclusions represented oxychromatic degeneration of chromatin rather than extraneous (virus) products and when we found them as a concomitant of burns he felt that this substantiated his view.

The Councilman lesion can hardly be regarded as a true cytoplasmic inclusion body, yet it is something of the same order and it, too, finds its chief association with virus diseases, namely, yellow fever and Rift Valley fever (Daubney *et al.*, 1931, Findlay, 1933). It is possibly a non-specific cytoplasmic degeneration, but like the nuclear changes, sufficiently distinctive to suggest some common ætiological factor underlying its various occurrences.

On the basis of the present study one can do no more than point out that the toxæmia in certain cases of burns produces an effect upon the liver similar to that observed in certain virus diseases and to suggest, therefore, that the noxious agent in both instances,

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- VONGLAHN, W C, AND PAPPENHEIMER, A M Intranuclear inclusions in visceral disease, *Amer J Path*, 1925, 1 445
- WELLS, H G Chemical pathology, 5th ed, *Philadelphia and London*, 1925, pp 651-654

a dome-shaped elevation of the skin; this part of the tumour seemed to be encapsulated and its appearance suggested an intracanalicular fibro-adenoma. Immediately beneath lobe A and continuous with it was a large firm mass of new-growth (B), of irregular and indefinite outline, which extended outwards towards the axilla and suggested scirrhus cancer, a very firm rounded nodule (C) attached to its inner margin appeared to be composed

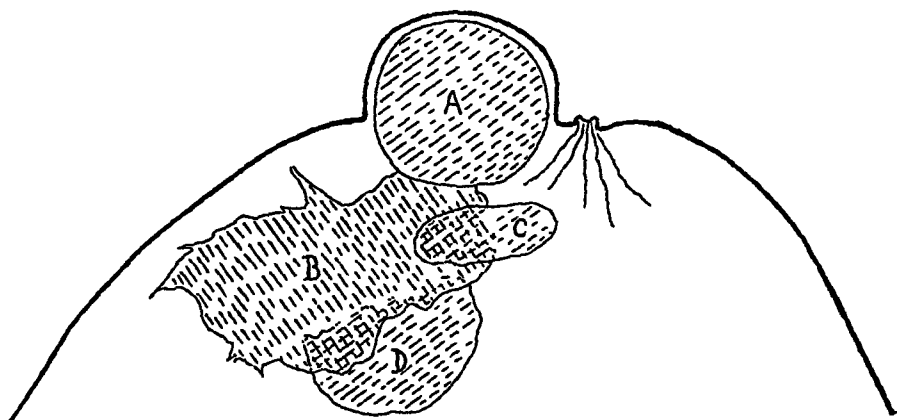


FIG 1 —Outline sketch of mammary tumour on section (reduced)

of dense fibrous tissue. From the deep aspect of the irregular mass (B) there projected a more or less hemispherical lobe of tumour tissue (D), showing central degeneration and softening but in its peripheral parts tough and fibrous, it was not encapsulated but showed an ill-defined infiltrating margin, and, while in the main of softer consistence than the intermediate tumour mass, contained in its outer parts several hard translucent nodules.

Histological examination

The superficial lobe of the tumour (A) presented the typical characters of an intracanalicular fibro-adenoma with a somewhat cellular stroma (fig 2). Sections from the underlying irregular main mass (B) showed for the most part what appeared to be a sarcomatous structure composed of fasciculated spindle cells (fig 3). It was clear, however, that the case could not be regarded merely as an example of adenosarcoma, for in contiguous blocks of tissue areas of frank scirrhus carcinoma were found (fig 4) as well as areas in which the two types of cellular growth were intermingled (fig 5). The firm nodule (C) at the margin of the main mass was composed of moderately cellular fibrous tissue recalling the stroma of a simple fibro-adenoma, it contained no gland spaces but was traversed here and there by elongated strands of cells of epithelial type (fig 6). In the hemispherical, deep-seated lobe (D) there

lobe possessed the fine reticular meshwork of such tumours, and the epithelial channels were as usual circumscribed by a condensation of reticular fibres forming the basement membrane

Discussion.

This growth might have been classed as a 'mixed tumour' of the breast with carcinomatous and sarcomatous changes occurring in the fibro-adenomatous elements, but our study of its architecture suggests that the varied appearances may be better explained as a diffuse infiltration of the stroma of an intracanalicular fibro-adenoma by carcinoma arising in its own epithelium. The significance of the cartilage is discussed below.

The analysis of the structure of this complex tumour raises the possibility of explaining on similar lines the production of other diffuse carcinomata of the breast. The work of Muir (1927, 1931, Muir and Aitkenhead, 1934) has established that the usual course of events in the production of an infiltrating carcinoma of the breast is initiated by a neoplastic proliferation of the epithelium of the ducts, so that their lumina become filled with masses of carcinomatous cells unsupported by stroma (intraduct carcinoma). The carcinomatous proliferation may involve the ducts over a wide area and may at length extend into the acini. Restrained for a time within the walls of the ducts, the proliferating cells may at length break through into the surrounding tissues, and this will presumably occur more readily through the more delicate walls of the acini. Infiltration of the general stroma of the breast thus results and the malignant epithelial cells, calling forth the usual reaction on the part of the connective tissue cells, become enclosed in alveoli by fibrous tissue.

The structure of a terminal duct may be represented schematically as in fig 8 (I). The subepithelial connective tissue internal to the elastica is of an extremely delicate, loose myxomatous texture. Fig 8 (II) represents the development of scirrhus cancer of the breast, the epithelial cells forming a solid mass within the duct have broken through the wall of an acinus (unsupported as a rule by elastica), and the advancing cells form alveoli in the general stroma. Fig 8 (III) represents the structure of an intracanalicular fibro-adenoma, its stroma formed, as Cheatele and Cutler (1931) have shown, by the subepithelial connective tissue. As the tumour grows larger, the elastica opposite the site of origin becomes stretched and ultimately disappears along with the other structures of the wall (fig 8, IV). If malignant proliferation were to commence in the epithelium of the adenoma (fig 8, IV, c), the proliferating cells, although on an epithelial surface, would be deep within the adenomatous tumour, so that

rather that these were areas in which the neoplastic cells had become enclosed in groups and finally isolated by a hyaline material (fig 14) so that the semblance of cartilage was produced. In certain areas the simulation was remarkably close (fig 13) but it was clear nevertheless that the cells in the lacunæ were the epithelial cells of the diffuse carcinoma, for all stages of their incarceration in the hyaline matrix could be traced.

Elsewhere there were areas of mucinoid degeneration of the stroma, forming tracts of homogeneous material in which there was no suggestion of cartilaginous structure, but where these merged into the cartilage-like tissue the matrix became more condensed and hyaline in appearance.

The staining reactions of the anomalous cartilage were as follows. It gave metachromatic staining with methyl violet, methylene blue, thionin (with which the most helpful preparations were obtained) and safranin, it stained selectively with mucicarmine, purpurin and bismarck brown—a reaction which Foulds (1937) and others have found of particular value for the demonstration of cartilage. On the other hand with mucin the same reactions were obtained, both in the areas of mucinoid degeneration above referred to and in control preparations of normal tissues. Mucin and the ground substance of cartilage are closely allied in physical and chemical characters and I have been unable to find any histochemical reaction by which they may be distinguished. This question does not ordinarily arise since the morphological criteria are in normal circumstances sufficient. Under pathological conditions, however, the distinction between true cartilage and pseudo-cartilage is not always clear, as in the case of mixed parotid tumours.

From critical analysis of the histological appearances in the present case it is impossible to escape the conclusion that one is dealing with a new-growth in which there has developed not only true cartilage and osteoid tissue, probably of metaplastic origin from the stroma, but also pseudo-cartilage from the isolation of epithelial cells in a hyaline matrix. This conclusion may be examined in the light of Foulds' recent experimental work, which has thrown a new light on the development of cartilage and bone in mixed tumours.

In a transplantable carcinoma of the oviduct of a domestic fowl he observed during transmission the elaboration of the structure of a "mixed" tumour. The original growth was purely carcinomatous but subsequently bone and cartilage were frequently present. The cartilaginous and bony tissues were not transplanted but always developed anew, only the epithelial component was neoplastic. In the production of tissue with the morphological characters of cartilage he found that two distinct processes were concerned. In the one, true cartilage was formed through metaplasia of the connective tissue cells of the stroma. In the other a pseudo-cartilage

PLATE LXII

- FIG 9 —Area from tissue bounding (D) at a point where there are indications of a duct wall. The tissues of the duct wall internal to the elastica are infiltrated by diffuse carcinoma. $\times 90$
- FIG 10 —Proliferation of the lining epithelium at one point in a glandular cleft (portion D). $\times 75$
- FIG 11 —Silver impregnation of the reticulum in a cellular, apparently sarcomatous, area. The cells lie in groups within the meshes of a reticular net. $\times 190$
- FIG 12 —Silver impregnation of the reticulum of a diffusely cellular area in which lie frankly carcinomatous alveoli. The reticulum forms a rich network with well defined open meshes. $\times 190$
- FIG 13 —Nodule having the morphological characters of cartilage. $\times 55$
- FIG 14 —At the margin of a cartilage-like nodule the diffusely infiltrating carcinoma cells become separated and surrounded by an amorphous hyaline matrix. $\times 190$

as in Foulds' tumour, to metaplasia of the connective tissue cells of the stroma

Summary

A complex malignant mammary tumour is described which contained areas of fibro-adenomatous, carcinomatous and apparently sarcomatous structure, together with nodules resembling cartilage

It is suggested that in an intracanalicular fibro-adenoma malignant transformation of the epithelial component led to diffuse carcinomatous infiltration of the stroma in which mucinoid degeneration was followed by the development of an epithelial pseudo-cartilage and by the formation of true cartilage and osteoid tissue by metaplasia of the stromal cells.

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No such effect was observed with 25 *gravis* and 25 *intermedius* strains tested in the same way. For these, heated blood provided a suitable enrichment. For convenience inhibited strains will henceforth be referred to as "sensitive" and those enhanced by heated blood as "resistant". With resistant strains even 20 per cent of heated blood had no effect except to reduce the size of the colonies.

TABLE I

Growth of 166 mitis strains of C. diphtheriae on heated blood agar

Description of growth	Number of strains	Virulence tested	Virulent	Non virulent
Rich confluent in 18 hours	29	25	20	5
Fine in 18 hours, rich confluent in 48 hours	26	11	6	5
Fine in 48 hours	17	13	10	3
Few colonies in 18 hours and under 200 in 48 hours	94	46	42	4
Total strains	166	95	78	17

Effect of heating solutions of lysed blood cells

In preliminary experiments it was found that the red cells were responsible for the inhibition and that serum counteracted it to some extent. Therefore in subsequent experiments only the cells were used.

The plasma was removed from fresh oxalated horse blood and the cells washed 3 times in 10 volumes of saline. The red cells were lysed by the addition of sterile distilled water up to the original volume of blood. Since the loss in centrifuging is small, the haemoglobin content of such a solution is approximately equivalent to that of whole blood and its concentration in media can thus be expressed in terms of whole blood and this has been done throughout this paper. In one experiment the solution was exposed to temperatures ranging from 60° to 120° C for 15 minutes, while in another the temperature was kept constant at 65° C and the time varied from 10 minutes to 1 hour. To avoid the formation of a coarse precipitate the solutions were further diluted 1:4 before heating and were well disintegrated before use by shaking and drawing up and down with a pipette. Calculated amounts of the heated solutions were added to melted agar at 50° C and plates poured. Control plates received the same volume of saline. All the plates were inoculated with a loopful of undiluted suspension and of dilutions 1:10, 1:100 and 1:1000 and examined at various intervals over 3 days. To see if the effect was specific the media were also inoculated with various strains of *H. influenzae* and *N. gonorrhoeae*. The effect of exposure to various temperatures for 15 minutes is illustrated in table II.

The two *mitis* strains were selected sensitive strains. Addition of unheated blood or lysed cells caused an enhancement of the speed of growth which was still evident after heating to 60° C.

at 100° C inhibition was marked after 1-2 minutes, whereas at 75° C it required 5 minutes and at 65° C 40-50 minutes to produce the same effect. Coagulation was correspondingly slower at the lower temperatures and at 65° C it could be seen to appear before the onset of inhibition. Moreover a partly coagulated solution was quantitatively less effective than one more completely coagulated, but once coagulation was complete further heating did not result in an increase of the inhibitory property.

TABLE III

Effect of heating lysed blood at 65° C for varying periods of time

Strain	Dilution used as inoculum	Number of colonies developing in 48 hours on agar containing							
		no addition	lysed blood heated at 65° C for (minutes)						
			0	10	20	30	40	50	60
<i>Mitis</i> 332	1 1000 Undiluted	95 PC	120 C	132 C	144 C	112 C	25 PC	2 15	0 6
<i>Mitis</i> 1156	1 1000 Undiluted	63 PC	85 C	70 C	100 C	28 PC	4 26	0 1	0 2
<i>Gonococcus</i> 3	1 1000	0	63	45	84	67	55	75	80
<i>H. influenzae</i> T	1 1000	0	100 (pin-point)	160	110	115	170	140	125

C = confluent

PC = partly confluent

Concentration of red blood cells = 4 per cent whole blood

The state of particulation of the coagulum greatly influences the result. Thus a coarse hard precipitate has less effect than a fine, easily dispersible one. This was shown by diluting solutions of red blood cells to the same extent with water and saline, heating them to 100° C for 15 minutes and incorporating in agar equal amounts of the resulting precipitates. The finer precipitates obtained from the watery solutions were more inhibitory than the coarse saline coagula, of which double the quantity may be required to produce the same effect. It was also found that a watery solution heated to 100° C for two minutes was more inhibitory than it was after addition of saline and further heating for 15 minutes to 1 hour, during which large coarse aggregates formed. Well washed red blood corpuscles, particularly in low dilutions (1 2 to 1 5), form coarser precipitates when heated in water or saline than do similar preparations to which a little serum or gelatin has been added. If blood is centrifuged and the plasma pipetted off and the unwashed corpuscles are laked with distilled water, the resulting solutions can be heated in as low a dilution as 1 2 without giving rise to coarse precipitates, indeed the coagula are too fine to be thrown down completely after one hour's centrifuging at 4000 *r p m* and will in large part pass through ordinary filter paper. With

Some experiments suggest that the heated blood has a certain radius of action. Heated blood agar plates were covered with layers of nutrient agar of different thickness (1 mm upwards). It was found that growth improved with increase in the thickness of the superimposed layer of agar. On a 2-3 mm layer it was generally fair but a layer of 1 mm made little difference. This suggests that the inhibitory substance is soluble and diffuses slowly into the agar above it, though the experiments on precipitates do not accord with such an explanation. If this were the case storage would allow more diffusion to take place and if a 2 mm layer of agar made growth possible immediately after preparation it might not do so two days later. Such an effect of storage has not been observed.

Effect of precipitation by acid or alkali

Two c.c. of *N* NaOH or *N* HCl were added to 10 c.c. of solutions of washed lysed blood. With the acid the solution blackened immediately and within 5 minutes was treacly in consistency, with the alkali it became bright red and slowly darkened on standing. The acid was allowed to act at room temperature for 5 minutes and the alkali for 1 hour. Both solutions were further diluted 1:5 and carefully neutralised, a precipitate forming. All supernatants were coloured to some extent, the alkaline always much more deeply than the acid, the alkaline was cherry red and the acid a brownish colour. The precipitates were washed by centrifugation in phosphate buffer solution at pH 7.6 until the supernatants were practically colourless and then made up with buffer to a suitable volume and broken up by drawing up and down in a pipette. Various amounts were added to nutrient agar and plates poured.

It was found that small amounts of both kinds of precipitate enhanced the growth of sensitive strains of *C. diphtheriae* but larger quantities inhibited them, whereas the growth of resistant strains was enhanced. It was difficult to compare quantitatively the activity of blood thus treated with that of heated blood, since precipitation was partial and the coagula coarse and the loss during washing was considerable, as a portion of the precipitate redissolved in the buffer solution. Approximately four times as much blood was required initially to produce the same effect after treatment as was required when heat was employed. On inhibitory concentrations of such preparations gonococci grew well and *H. influenzae* also flourished if yeast extract was added or in proximity to a staphylococcus colony, but without the addition of "V" factor growth was generally poor or absent.

Washed precipitates when soaked for 24-72 hours in buffer produced supernatants which were not inhibitory, and broth in which precipitates were soaked overnight was not found inhibitory when the precipitate was removed. The precipitate, however, remained inhibitory after such treatment. The results are in all respects similar to those obtained with heated blood.

test-tubes incubated aerobically at 37° C. The level at which reduction occurred was different in different batches of agar. The addition of fresh or heated blood had little effect on the result, though tending on the whole to increase the reducing power of the medium. No evidence was found of any tendency to increase its oxidising power.

Effect of potassium cyanide on inhibition by heated blood

Cyanide itself is inhibitory to the growth of *C. diphtheriae*. It had a marked inhibitory effect on agar shake cultures of the sensitive strains used in the above experiments in a concentration of 0.01 per cent. and a noticeable influence at 0.001 per cent. On plate cultures its effect appeared to be less and was somewhat irregular, generally 0.02 per cent. of a fresh solution was sufficient to inhibit inocula of a thousand viable organisms if the plates were incubated in a closed container or wrapped in cellophane. All solutions of cyanide were made up on the day of the experiment and were adjusted to pH 7.2-7.6 with HCl.

When 0.005-0.1 per cent. was added to melted agar containing 5 per cent. of heated blood the media were as unsuitable for growth as those containing no cyanide, but the same strains grew well on fresh blood plates containing concentrations up to 0.05 per cent. The effect of the cyanide was, however, very striking when fresh laked corpuscles were coagulated in the presence of various concentrations of KCN by mixing in watery solution or in melted nutrient agar and heating the mixture at 65° C. for 1-2 hours. With the equivalent of 5 per cent. of blood, growth was completely restored by 0.02-0.06 per cent. of KCN but not by higher or lower concentrations. It was observed that blood heated at 65° C. in the presence of KCN was incompletely coagulated in 2 hours, whereas in its absence coagulation was practically complete in 1 hour.

When blood-cyanide mixtures were heated at 65° C. for 1 hour, either in the medium or in water, and then at 100° C. for 2 minutes, practically complete coagulation took place, the coagula being coarser than in the control tubes containing no KCN. No growth-restoring action was now observed with any concentration of cyanide. Coagulation at 100° C. without preliminary heating at 65° C. gave the same result. A fine precipitate was obtained by heat coagulation of lysed blood and was mixed with KCN in such a way that the concentration of precipitate was equivalent to blood diluted 1:3 while the KCN concentration ranged from 0.03 to 0.3 per cent. In the presence of cyanide, precipitation occurred in 24 hours and the supernatants acquired a red colour which deepened on standing, whereas in the absence of the salt

and Mirsky (1925, 1925-26, 1928-29, 1929-30) have shown that hæmoglobin behaves like other proteins under the influence of coagulating agents, that heat, acid and alkali denature it and that the denatured hæmoglobin is precipitated near the neutral point. The coagulated protein consists of a combination of hæm with denatured globin. If coagulation is carried out in the presence of reducing agents, the hæm of the denatured conjugated protein remains reduced (as in hæmoglobin) and the resulting product is described as globin hæmochromogen. This is easily oxidised on exposure to air and is converted into hæmatin which is also known as protohæmatin or catahæmoglobin. The denaturation of oxy-hæmoglobin results in the formation of hæmatin but sodium hydrosulphite can reduce this to globin hæmochromogen. Thus hæm when linked to denatured globin is much more easily oxidised than when in combination with the native protein. We may suppose that under the conditions of preparation of our coagula, which was always carried out in the presence of air, catahæmoglobin (hæmatin in Anson and Mirsky's terminology) was obtained. If this product were inhibitory, addition of sodium hydrosulphite or anaerobic incubation may have reduced it to globin hæmochromogen and removed the inhibitory property. In connection with this point, it should be mentioned that Anson and Mirsky (1925) state that mere removal of oxygen did not cause the reduction of hæmatin to hæmochromogen, but in our experiments the reducing capacity of the medium was also in play. It may thus be that the explanation of our findings lies in the fact that the iron of hæmoglobin and of hæmochromogen is ferrous whereas that of catahæmoglobin is ferric and that it is the latter which inhibits. Whether it must be contained in the pyrrole structure of hæm linked to a denatured protein we do not know. It must be borne in mind that our inhibitory substance was not in solution and therefore an imitation of the effect with salts of iron should be attempted under the same conditions. With regard to the mode of action of the inhibitory substance, an interference with some essential enzymes may be postulated. This possibility invites experiments with the Barcroft and Thunberg techniques on the respiration of sensitive strains of *C. diphtheriae*.

The effect of hydrosulphite and of anaerobic incubation might be due to something other than the reduction of hæm in catahæmoglobin. By lowering the oxidation-reduction potential of the medium the enzymes of the experimental organisms may have been placed under a set of conditions where they were no longer sensitive to the action of coagulated hæmoglobin, whether in the form of globin hæmochromogen or catahæmoglobin, in other words it may not have been the reduction of the inhibitory substance which mattered but the changed behaviour and sensitivity of the

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of nephritis In order to do this the clinical diagnosis, whenever possible, has been subjected to confirmation at autopsy The nature of this change and its general significance are not dealt with

Samples of venous blood were collected, using a syringe with a relatively wide-bore needle (no 12), and allowed to clot in previously autoclaved test-tubes for 4-5 hours before making the test In this, 0.1 c.c. of the serum so obtained was added to 1 c.c. of a 3 per cent suspension of sheep red blood cells These had previously been washed and suspended in 0.85 per cent saline and sensitised by the addition of 0.1 c.c. of standard immune body (Burroughs Wellcome) to every 20 c.c. of the suspension The mixtures were shaken and placed in an incubator at 37° C for one hour They were then centrifuged at approximately 3000 *r.p.m.* for three minutes 0.5 c.c. of the supernatant fluid was added to 1 c.c. of decinormal HCl, and the hæmoglobin present estimated exactly five minutes later in a Zeiss hæmometer This instrument expresses its results in terms either of grammes or of percentages, we adopted the latter

By this method the complement titre of the blood is expressed in terms of the amount of hæmolysis occurring in a given system under standardised conditions In order to make the results of successive experiments comparable the suspension of cells was so adjusted that complete hæmolysis gave a reading of approximately 100, and the figures throughout are given as a percentage of complete hæmolysis Over 400 estimations have been carried out by this method on sera from various sources and the readings have, in general, ranged from 70 to 100 per cent, the average reading being about 85 per cent In rheumatic fever and chorea lower readings were the rule, ranging from 60 to 80 per cent, and in one instance a reading as low as 22 per cent was obtained In only two instances so far, apart from the cases of nephritis under review, has a reading in the region of zero been obtained, one was in a case of fulminating eclampsia, the other in a case of malignant endocarditis

The material forming the basis of the present paper consists of estimations, often repeated, of the complement in 38 cases of nephritis The classification of nephritis is as complicated and difficult as any in medicine, and it was thought desirable in the first place to use as simple a classification as possible, of necessity a clinical one, as relatively few of the cases were submitted to post-mortem examination The cases were divided into three main groups, acute glomerulo-nephritis, subacute nephritis and chronic nephritis Six cases did not fall readily into any of these groups and are considered separately The diagnosis of acute glomerulo-nephritis in a child following scarlet fever or tonsillitis is usually made with little difficulty and with a fair degree of certainty In older subjects it is sometimes difficult to determine if the attack is one of primary acute nephritis or a flare-up of an older lesion A history of past acute nephritis is so often lacking

the onset) was still not quite normal, but the boy appears to have made a good recovery

Case 9 Complement at the 4th week was still causing only 36 per cent hæmolysis, she was taken home and died three weeks later of uræmia

Case 10 Complement at the end of 3 months was still causing only 60 per cent hæmolysis, he died a year later of uræmia

TABLE I

Cases diagnosed as acute glomerulo-nephritis

No	Sex	Age (years)	Blood pressure	Blood uric acid (mg per 100 c c)	Blood complement in terms of percentage hæmolysis	Remarks	After history
1 W J W	M	3½	130/	138	95	Not acute nephritis See text (p 523)	Died, P M 62/38
2 M E C	F	7	120/80	70	5	Complement normal (100) in 5 weeks	Recovered
3 P O C	M	9	164/120	32	42	Very mild case	Recovered
4 H W	M	10	131/90	136	8	Complement normal (85) in 6 weeks	Recovered
5 T H	M	10	138/95	338	0	Complement still 0 after 10 weeks	Died, no P M (see text)
6 D U	M	11	150/110	41	32	Complement normal (75) in 5 weeks	Recovered
7 D N	F	12	130/70	135	25	Complement still low (20) 2 months later	See text
8 A T	M	12	125/98	72	5	Complement still low (60) 2 months later	Recovered (see text)
9 M A	F	12½	130/106	64	15	Complement still low (36) 4 weeks later	Died (see text)
10 P B	M	13	165/125	50	20	Complement still low (62) 3 months later	Died (see text)
11 F B	M	13	160/100	132	18	See text (p 524)	Died, P M 182/37
12 J S	M	15	170/110	95	4	Complement normal (96) in 4 weeks	Recovered
13 C L	M	15	180/100	87	2	Complement normal (80) in 4 weeks	Recovered
14 G E	M	17	160/170	33	0	One estimation (on admission) only	Recovered
15 J B	M	19	190/130	46	20	Complement normal (80) in 14 weeks	Recovered
16 W C	M	19	145/95	200	80	Not acute nephritis See text (p 523)	Died, P M 181/38
17 F B	M	41	200/120		0	One estimation only	Died, no P M
18 R N	M	51		360	10	See text (p 524)	Died, P M 226/37

While the persistent deficiency of complement may prove to be of some prognostic value, it must be admitted that there was adequate clinical evidence that these cases were passing into the subacute stage, and a bad prognosis would have been given on these grounds alone

Four cases which died in the acute stage were examined *post mortem* and it is fortunate that these include the two cases, clinically acute nephritis, which did not show reduction in complement

has been noted occasionally in nephritis (as in case 18 of this series) and a glomerulitis is often noted in cases of polyarteritis nodosa. The association of all three in one case is rare, but an example very similar to ours has been reported by Helpern and Trubek (1933), where the endocarditis was of gonococcal origin and presumably primary. There is no point in attempting to discuss here which of the three lesions in our case was the primary, it is sufficient to stress that the nephritis was not in the active stage.

Case 11 F B, a boy aged 13, was admitted suffering from acute nephritis and peripheral neuritis of both arms. The neuritis subsequently extended to the legs and he died suddenly apparently of a diaphragmatic palsy.

Summary of post-mortem findings P M 182/37 Slight œdema of subcutaneous tissues, excess of fluid in pleural and peritoneal cavities. *Kidneys* considerably enlarged, with broadened cortices, in which glomeruli can be seen as slightly projecting bloodless pin-points. The histological picture is that of an active acute glomerulo-nephritis with some early subacute features, there are no signs of older renal disease and all the glomeruli appear to be affected. In the ulnar and sciatic nerves the changes are quite marked in paraffin sections. In places the nuclei have disappeared and the degenerated nerve fibres are swollen and dilated and often spirally twisted in corkscrew fashion.

We have been unable to discover in the literature any recent description of peripheral neuritis complicating acute glomerulo-nephritis. The cases described by Leyden in 1880 (quoted by Ross and Bury, 1893) and Dixon Mann in 1887, however, seem to us to resemble sufficiently the case under discussion to warrant our considering that both the nephritis and the polyneuritis were due to the same underlying cause.

The other fatal case, confirmed at autopsy, was the following.

Case 18 R N, a man aged 51, was admitted in a semi-comatose condition with slight œdema and a blood urea of 360 mg per 100 c c.

Summary of post-mortem findings P M 226/37. Œdema of subcutaneous tissues, excess of fluid in peritoneal cavity, acute fibrinous pericarditis, uræmic hæmorrhages and ulcers in colon. *Kidneys* (22 oz) have the appearances of a subacute nephritis in an early active stage. The lesions are mainly intracapillary, but crescents are not rare. The activity of the process is indicated by occasional fibrinous thrombi in the capillary loops and numerous polymorphs in and around the glomeruli. An unusual finding is the presence of necrotising arteritis in a few medium-sized and smaller arteries.

Group 2 Cases diagnosed as subacute nephritis

All four cases in this group had a history of acute glomerulo-nephritis less than a year before admission, with persistence of renal signs and symptoms, mainly œdema, albuminuria and high blood urea. The complement, while not at the low levels of the acute cases, was reduced, the figures being 74, 70, 52 and 64. Three

the cases by convulsions. There were, however, differences sufficiently marked to exclude their being labelled acute nephritis. The average age was much greater, the duration longer, uræmic signs were more prominent and albuminuric retinitis was generally present. In every case the blood complement was well within normal limits. The prognosis was much worse than in the acute

TABLE II
Cases diagnosed as chronic nephritis

No	Sex	Age	Blood pressure	Blood urea (mg per 100 c c)	Blood complement in terms of percentage hæmolysis	Remarks
1 J E R	F	26	220/	336	85	Died, no P M
2 J A	M	27	210/170	240	98	Died, P M 13/38, chronic nephritis
3 J S	M	32	195/110	305	93	Discharged moribund
4 G K	M	34	218/118	168	98	Died, P M 192/38, chronic nephritis
5 T S	M	37	180/110	330	92	Died, P M 136/38, late subacute nephritis
6 J H	M	38	195/70	192	90	Died P M 246/38, late subacute nephritis
7 W J W	M	38	240/130	264	90	Discharged, no improvement
8 J P H	M	42	210/155	172	95	Discharged, no improvement
9 J W M	M	57	210/132	212	86	Died, no P M
10 W J B	M	66	155/125	168	82	Discharged, no improvement
11 R B	M	49	240/150	58	80	Died, essential hypertension, no P M
12 W S	M	25	210/130	144	95	Died, P M 395/37, essential hypertension

cases, 6 of the 10 died, and in four the diagnosis was confirmed *post mortem*. Both the cases of essential hypertension died and one came to autopsy. Details of these five cases are given below.

Case 2 J A, a male aged 27, was admitted with three weeks' history of headaches and polyuria. No previous history of acute nephritis. Albuminuric retinitis present.

Summary of post-mortem findings P M 13/38. No œdema, marked left ventricular hypertrophy, uræmic hæmorrhages in ileum. *Kidneys* (6 oz) reduced in size, with appearances of chronic nephritis. Microscopical examination confirms the lesion as a severe chronic nephritis with no special features.

Case 4 G K, a man aged 34, was admitted with polyuria, albuminuria and failing vision. He had had acute glomerulo-nephritis five years before and marked albuminuric retinitis was present.

Summary of post-mortem findings P M 192/38. No œdema, great hypertrophy of left ventricle. *Kidneys* very small, capsules adherent and external surfaces granular. Section showed a uniformly narrowed fibrotic cortex with loss of differentiation from medulla. Microscopically a chronic glomerulo-nephritis of severe degree is present with no evidence of a fresh lesion or of anything indicating a flare-up of the old.

These cases might represent the so-called nephrotic type of chronic glomerular nephritis, though it is equally possible that certain of them in view of their short histories might be mild

TABLE III.
Nephritis of doubtful nature

No	Sex	Age	Blood pressure	Blood urea (mg per cent)	Blood complement in terms of percentage hemolysis	Remarks
1 A C	M	18	130/90	32	75	One month before admission transient attack of abdominal pain and hæmaturia, slight œdema of ankles and face, renal efficiency tests normal, albuminuria
2 W J	M	25	135/105	80	82	Five weeks before admission sore throat, two weeks later transient œdema with hæmaturia, recovered completely within the week, slight albuminuria
3 H M	M	25	150/95	68	75	Acute tonsillitis 3 years prior to admission, 10 weeks ago sore throat and rheumatic pains, 7 weeks ago transient œdema of feet, albuminuria
4 W T	M	33	130/90	42	80	Cold 8 weeks ago, transient œdema of feet 7 weeks ago, with hæmaturia, on admission gross albuminuria only
5 S R	M	34	120/85	34	94	Two months before admission otitis media, 3 weeks later septic finger followed 1 week later by generalised œdema with "cloudy urine", admitted with slight generalised œdema and gross albuminuria
6 E H	M	37	165/100	22	95	Three months before admission generalised œdema and marked albuminuria, otherwise well, normal renal function tests

examples of acute nephritis admitted too long after the onset to show the typical reduction in complement. It is interesting that, despite the very heavy loss of protein in the urine, the complement remained normal.

Discussion

Our work over the past three years would seem to show that in certain cases of nephritis the complementary activity of the serum is low in comparison with that of normal individuals as shown by its lytic activity for sensitised sheep cells.

Scheller (1910), Thiele and Embleton (1914-15) and later workers have shown that the rate of hæmolysis by complement becomes slower as it approaches completion. Methods employing complete hæmolysis as an end point tend therefore to lose in sensitivity, and

the complement might well have returned to normal by that time, as in cases 2, 6, 12 and 13 in table I. In cases 3 and 6 there is a history suggestive of renal disease months before admission and, of the other cases in the series, no 5 is more suggestive of toxic nephrosis than of acute glomerulo-nephritis.

So far as our work is concerned it would seem that a considerable deficiency in the complementary activity of the blood is, in a case of nephritis, pathognomonic of those processes producing acute glomerulo-nephritis. It has, then, a certain practical diagnostic value, and it is apparent that even in our limited series the estimation of the complement proved itself in retrospect to be of diagnostic value in cases such as nos 1 and 18. It may also be that it has some ætiological significance, which if established would add greatly to its diagnostic value.

It would seem possible that this reaction as it stands may prove capable of answering the question whether chronic glomerulo-nephritis is usually the result of a single progressive attack or whether relapses commonly occur. Boyd (1935) is of the opinion that this question also can "only be answered from analogy," and states that "In nephritis also there is often clinical evidence of repeated exacerbations of the disease, which probably reflect successive blows to the already damaged kidney, so that once more the damage of the end stage may be extreme." Fishberg (1931) thinks that true relapses occasionally occur, and both authors quote the same case, that reported by Mann in 1887. In our admittedly small series we found no serological evidence of a true flare-up in the cases of chronic nephritis, although some of them were admitted with signs of acute renal insufficiency, and with the exception of an example of chronic focal nephritis (*nephritis repens* of Russell) none of the chronic cases examined at autopsy had any morbid anatomical evidence of recent activity in the kidneys.

While further work is necessary to determine the significance of this low complementary activity of the blood, our results fall readily into line with those of the earlier workers summarised at the beginning of this paper. In the earlier literature it was advanced as a pathognomonic sign of uræmia, and when this was disproved by Senator and subsequent workers the phenomenon appears to have fallen into ill-deserved neglect and to have been virtually forgotten. Had a distinction been made between the so-called uræmic fits associated with acute glomerulo-nephritis and those with terminal renal failure, it is probable that this would never have happened. Further work would have quickly shown that it occurred in acute glomerulo-nephritis only, whether accompanied by fits or not.

auricular wall by a layer of thrombus, but there was a cleft-like space leading to the mitral orifice between the auricular septum and the tumour. There was commencing invasion of the wall of the aorta where it crossed the left bronchus, and nodular metastases were present in the lower lobe of the left lung.

Mead (1932-33) reports a bronchogenic carcinoma of the right lung in a male aged 65. A secondary extension had entered and grown within one of the right pulmonary veins as far as the left auricle, which was distended with tumour, the lower portion extending through the mitral orifice into the left ventricle. There was no invasion of the endocardium.

A primary spindle cell sarcoma of the right lung in a male aged 18 is recorded by Johns and Sharpe (1935). The lumen of the right pulmonary vein had been invaded and the left auricle was practically filled by a polypoid mass of tumour which was causing mitral obstruction. The tumour mass lay free in the auricular cavity, but there appeared to be invasion of the intima of the vein. Nodular metastases were present in the left lung and liver.

The following cases of new growth within the left auricle form a group whose characters differ from those of the first series.

Retrograde invasion of the left pulmonary vein by a primary myoma of the left auricle is recorded by Justi (1896). The tumour took the form of a bilobed nodular polypoid mass, the larger portion of which originated from the medial wall of the auricle at the level of the attachment of the mitral cusps. A secondary extension passed into the ostium of a left pulmonary vein and could be demonstrated on section of the left lung. No evidence of new growth in other organs.

Muller (1932) describes a pedunculated fibrosarcoma of the left auricle in a female aged 68. It almost filled the auricular cavity and obstructed the ostia of the pulmonary veins and the mitral orifice. A polypoid lobulated portion passed into a right pulmonary vein and became continuous with a sharply circumscribed mass about the size of a goose's egg in the middle lobe of the right lung. There was a sharp line of demarcation between tumour and auricular endocardium except in one area where the two were separated by a zone of granulation tissue. Nodular metastases were present in the left lung.

A primary spindle cell sarcoma of the heart in a male aged 34 is described by Baldwin (1910). The right ventricle was grossly dilated and a large pedunculated tumour almost filled the cavity of the left auricle. It originated from the margin of the ostium of the left inferior pulmonary vein and was encapsulated.

Passler (1896) reports a case of bronchial carcinoma in a male aged 52 which may conveniently be included in this series although the lumen of the pulmonary vein was not involved. The main branch of the right pulmonary artery had been eroded and the pulmonary veins were compressed. There had been widespread dissemination in the lymphatic plexus surrounding the pulmonary veins, and while strands of tumour cells were found in the walls of the veins, the intima had not been perforated. The growth accompanied the left pulmonary veins into the wall of the left auricle.

We have observed two further examples of neoplastic invasion of the pulmonary veins and left auricle.

pulmonary vein were plugged by growth close to the lung root. The lower division of the right branch of the pulmonary artery was compressed by the surrounding growth. The extra-pulmonary portions of the veins draining the upper and middle right lobes were partially thrombosed, and the superior and inferior right pulmonary veins were distended with growth to a diameter of about 3 cm. The growth in the superior pulmonary vein did not extend further than the junction of the vein and its tributaries from the upper and middle right lobes. The left auricle contained a tumour mass about the size of a tangerine orange. It was continuous with the tumour invading the right pulmonary veins, but neither the auricular appendage nor the left pulmonary veins were invaded. A polypoid portion passed through the mitral orifice causing bulging displacement of the mitral cusps. The auricular wall could be detached from the periphery of the growth, and there was no evidence of invasion of either mural or valvular endocardium. The myocardium was rather pale and soft and there was slight hypertrophy and dilatation of the right ventricle. There were no growths in the abdominal viscera, which showed only chronic passive congestion.

Anatomical diagnosis Bilateral metastatic pulmonary sarcoma, invasion of right pulmonary veins, left auricle and mitral orifice, hydrothorax, congestive cardiac failure.

Microscopical examination

Portions of tumour from the lower lobe and hilum of the right lung, subpleural deposits from the left lung and the tracheo-bronchial and right broncho-pulmonary lymph nodes were fixed in 10 per cent formol-saline. The stains used were hæmatoxylin and Biebrich scarlet, Masson's hæmatoxylin, fuchsin ponceau and light green, and Foot's modification of the Del Río-Hortega silver carbonate method. Further sections of the leg tumour removed in 1930 were similarly stained.

Lower lobe of right lung The tumour is composed of sheets of monomorphic polyhedral cells (fig 2) irregularly interspersed by numerous capillaries and occasional coarser fibro-vascular strands. The cells measure 8-10 μ in their largest diameter. The nuclei are oval or spheroidal with sharply defined nuclear membrane, and the chromatin has a vesicular arrangement. Nucleoli are ill defined, mitoses average 5 per high power field. The cytoplasm is pale and poorly defined, in dividing cells it is somewhat acidophilic. There is no evidence of fibrillary intercellular stroma and reticulin fibrils are limited to the capillary walls (fig 3). There is, however, an intercellular matrix of minute faintly acidophilic granules (figs 2 and 3), which often occur in pericellular clusters.

PLATE LXIII

- FIG 1 —Case 1 Secondary reticulosarcoma of lung invading left auricle The right lung has been bisected and the posterior half reflected outwards from the hilus An invaded branch of the inferior right pulmonary vein is seen in cross section below the main hyparterial bronchus, and the distended superior and inferior right pulmonary veins lie between the edge of the lung and the left auricle The posterior part of the coronal sulcus and the left auricular appendage have been removed so as to show the intra-auricular growth, which also presents at the orifices of the left pulmonary veins
- FIG 2 —Reticulosarcoma Metastasis in right lung, showing sheets of tumour cells interspersed by capillaries Granular material is present between the tumour cells Hæmatoxylin and Biebrich scarlet $\times 480$
- FIG 3 —Reticulosarcoma Metastasis in right lung, showing reticulin fibrils limited to capillary walls, and intercellular granular material Foot's stain $\times 300$

fibrosis of both lungs with dimming of the right base and (?) pulmonary collapse. The general condition gradually improved but the collapse did not clear up. In a month's time he was able to get up and could eventually walk three miles.

He was readmitted on 19th April 1937. During the previous six weeks dyspnoea had recurred and oedema of the legs had appeared. At the same time generalised pulmonary osteo-arthritis developed and cough with occasional hæmoptysis became troublesome. He was orthopnoic and slightly cyanosed, and the right chest was practically immobile. This area was characterised by stony dullness with absent V.R. and breath sounds. The apex beat was behind the sixth rib four inches from the mid-sternal line. The heart sounds were accentuated, with a variable apical systolic murmur. The dyspnoea and distress increased and the patient died suddenly on 5th May 1937.

Autopsy

Body poorly nourished. marked clubbing of fingers and toes. No gross abnormalities in upper digestive or respiratory tracts. Each pleural sac contained about two pints of clear yellow fluid. There were fibrous adhesions at the right apex and over the lower and outer surfaces of both lower lobes. There was a small hæmatoma in the right superior interlobar fissure. The tracheo-bronchial nodes were invaded by firm creamy white growth and the right main bronchus was slightly narrowed 1.5 cm. below its origin. The lumen of the main hyparterial bronchus to the right lower lobe was patent for 2 cm., but at this point it merged into and became surrounded by a mass of new growth (fig. 6) which extended in an antero-posterior and lateral direction almost to the diaphragmatic surface. The growth had an irregularly lobulated arrangement and was yellowish white in colour with numerous areas of necrosis and hæmorrhage. The branch of the superior pulmonary vein from the right middle lobe was thrombosed, while the right inferior pulmonary vein was distended by new growth to a diameter of 2.5 cm. The lower branch of the right pulmonary artery was compressed by growth. The remainder of the right lung was collapsed and cedematous, the middle lobe atrophic and moulded over the surface of the lower lobe. There was an obsolete fibro-caseous tuberculous focus at the right apex attended by slight bronchiectasis. The upper lobe of the left lung was cedematous, the lower lobe partially collapsed and its main bronchus plugged by mucopurulent secretion. The cavity of the left auricle was distended to a diameter of 10 cm. by a globular mass of growth which was continuous with the extension of the pulmonary tumour via the right inferior pulmonary vein. The intra-auricular growth was partially covered by a layer of thrombus and the orifice of the right superior pulmonary vein was partially occluded by thrombus. A probe could be freely passed between the growth and the wall of the auricle except in the superior and right lateral regions near the entrance of the dilated inferior pulmonary vein, where the

filled by extremely necrotic tumour and the adventitial region is surrounded by growth except in one area where there is a narrow zone of collapsed lung alveoli. The tumour cells are closely applied to the intima and in this region the large multinuclear cells tend to arrange themselves in a palisade-like manner. The wall of the vein is diffusely infiltrated by mononuclear leucocytes, there is no evidence of invasion of the perivascular lymphatics. The pulmonary artery is normal, and the hyparterial bronchi are not invaded at this level.

Wall of left auricle At the junction of the inferior right pulmonary vein and the auricular wall the tumour cells are closely applied to the intima. There is no evidence of invasion of the vein wall, but in places the endothelium is disorganised by a narrow zone of fibroblastic proliferation with capillary sprouts, which separates tumour and subintima. The relationship between tumour and mural endocardium (fig 10) is similar, but laminated thrombus is moulded over parts of the surface of the tumour and in some areas this thrombus widely separates tumour and heart wall. There is a slight amount of organisation of the thrombus from the endocardial aspect. There is no evidence of invasion of the muscle and generally there is marked thickening of the subendocardial connective tissue. In places this fibrosis encroaches on the myocardium and includes areas of granulation tissue. The visceral pericardium shows early organisation of a simple inflammatory exudate.

Tracheobronchial node There is diffuse invasion by necrotic spheroidal and spindle cell growth. The tumour cells are smaller than those in the lung and giant forms are not apparent.

Metatarsal bones. There has been extensive subperiosteal formation of rarefied cancellous bone, and between the spicules the loose connective tissue is cedematous and highly vascular. A certain amount of osteoclastic resorption is in progress.

Histological diagnosis Rhabdomyoma sarcomatodes

DISCUSSION

It seems clear that the pathological manifestations of the extension of pulmonary neoplasms into the left auricle via the pulmonary veins should be considered separately from cases of direct transpericardial proliferation of mediastinal neoplasms or of hæmatogenous cardiac metastases from a distant organ. Examples of the latter types of invasion of the left auricle have been recorded by Napp (1905), Yater (1931), Achard (1933), Matthews (1935-36), and McNamara *et al* (1937). According to Miller (1936) lymphatic permeation accounts for a limited number of cases of metastatic cardiac growth. It is possible moreover

and Zipkin (1907) but in both these cases the tumours were essentially teratomatous. Friedmann (1929) describes a nodular tumour involving both lungs and the parietal pleura in a male aged 59. There were metastases in the regional lymph nodes and abdominal viscera, and the tumour was regarded as originating in the right lung. Microscopically a fasciculated arrangement of spindle and giant round cells was apparent and while the morphology of these cells was entirely consistent with an origin in striated muscle, cross striation was not observed. The absence of the latter was regarded as an expression of cellular undifferentiation. In our case the tumour displays the morphological characters accorded to malignant rhabdomyoma by Cappell and Montgomery (1937) and we suggest that it may have originated through dislocation of a portion of the paraxial mesoderm into the splanchnopleure mesoderm.

In conclusion some reference may be made to the circulatory disturbances which may follow neoplastic invasion of the left auricular cavity. In both our cases there was mitral obstruction, but the gross encroachment on the auricular cavity appeared to cause relatively little circulatory embarrassment until the terminal stages. Muller states that the metastases in the left lung in his case (*vide supra*, p. 534) are probably to be accounted for by alterations in the venous currents in the left auricle due to the presence of the intra-auricular growth, with stasis in the left pulmonary veins and retrograde transportation of tumour cells. Metastasis via the bronchial arteries seems to be a more reasonable explanation of growth in the contra-lateral lung.

Mead (1932-33) refers to the so-called cardinal signs of cancer of the heart—cyanosis, precordial pain and frequent recurrent hæmorrhagic pericardial effusions, but states that almost invariably the symptoms are obscured by the primary disease picture. When they are observed they are generally attributed to the primary neoplastic condition and neglected. Yater (1931), on the other hand, draws attention to criteria suggestive of malignant mitral obstruction, among which are the absence of any history of rheumatic infection, progressive congestive failure and change of physical signs with alteration of position. A case of pedunculated thrombus of the left auricle which simulated mitral stenosis is recorded by Kaplan and Hollingsworth (1935). So far as could be determined clinically there was nothing suggestive of mitral obstruction in either of our cases apart from the variable apical systolic murmur in case 2.

SUMMARY

The literature relating to neoplastic invasion of the pulmonary veins and left auricle is reviewed and two new cases are described,

production (Panton and Valentine, 1932) was slight and was removed by filtration. The pathogenicity for normal rabbits inoculated intravenously with washed cocci from 18-hour broth cultures is shown in table I.

TABLE I

Results of infecting normal rabbits intravenously with Staphylococcus pyogenes, strain S 11

Dose of culture	No infected	No dying	Mean time to death in days	Staphylococci recovered from heart blood after death		Staphylococci not recovered from heart blood after death	
				No	Mean time to death in days	No	Mean time to death in days
1 c c or more	32	32	6.4	20	5.0	12	8.3
0.5 c c	29	27	9.1	12	8.7	15	9.1
0.1 "	5	3	9.7	2	8.0	1	13.0
0.01 "	3	3	13.0	0		3	13.0

Preparation of vaccines

Vaccine A An 18-hour broth culture was treated with 0.4 per cent formalin for an hour at 37° C and overnight at room temperature. It was tested for sterility and kept at 4° C. Before use the organisms were centrifuged and re-suspended in broth.

Vaccine B This was similar to A except that three-hour cultures in 0.05 per cent glucose broth were used.

Vaccine C Three-hour cultures in 0.05 per cent glucose broth (which according to Lyons should contain capsulated cocci) were placed in a water-bath at 37° C which was then rapidly brought to the boil, the culture was immediately cooled under the tap and used on the same day.

Vaccine D A three-hour glucose broth culture was heated at 60° C for exactly 10 minutes, cooled quickly and used on the same day. Heating for 10 minutes at 55° C reduced the viable organisms to about one millionth, at 60° C the culture was sterilised.

Vaccine E A three-hour culture of *Pasteurella pseudotuberculosis* in glucose broth was heated for 10 minutes at 60° C, which sterilised it.

Rabbits The initial weight was usually between 1750 and 2250 g, the heavier animals usually serving for controls. During the experiments they were weighed daily.

Test for immunity

Before each experiment the strain was passed through a rabbit and cultured in broth for 18 hours. The organisms were centrifuged, re-suspended in broth and diluted 1:2. The dose was 1.0 c c given intravenously, it contained an average of 269×10^6 living cocci. The exceptions to this dosage are specified.

Blood cultures

Blood was obtained from a marginal ear vein. The first few drops were discarded since they were much more heavily infected than the freely circulating blood. The blood was added to citrate broth. Amounts representing 0.5 c c of blood and tenfold dilutions were plated in duplicate.

TABLE II

Titre of antibodies during the course of infection with Staphylococcus pyogenes and results of cultures from the tissues (experiment IV)

Rabbit no	Immunised with vaccine	Time in relation to infection	Titre of antibodies			Result of culture at necropsy	
			Agg	AT	AL	Heart blood	Kidneys
92	C	Before	1 160	<0 05	<0 005	Sterile	One positive
		After 7 days	1 160	<0 05	<0 005		
		" 15 "	1 320	<0 05	0 005		
		" 22 "	1 160	0 1	0 08		
		" 28 "	1 80	1 0	0 01		
95	C	Before	1 80	<0 05	<0 005	Sterile	Both positive*
		After 7 days	1 80	<0 05	<0 005		
		" 15 "	1 40	<0 05	0 08		
		" 16 "					
109	B	Before	1 320	<0 05	<0 005	Sterile	One positive
		After 7 days	1 320	<0 05	<0 005		
		" 15 "	1 320	<0 05	0 16		
		" 22 "	1 160	6 0	0 16		
		" 28 "	1 40	3 0	0 16		
114	A	Before	1 40	<0 05	<0 005	Positive	Both positive†
		After 7 days	1 40	<0 05	0 005		
116	A	Before	1 20	<0 05	<0 005	Sterile	Sterile
		After 7 days	1 40	<0 05	<0 005		
		" 15 "	1 40	<0 05	<0 005		
		" 22 "	1 40	<0 05	<0 005		
		" 28 "	1 10	<0 05	<0 005		
124	Control	Before	<1 1	<0 05	<0 005	Sterile	Both positive
		After 7 days	<1 1	<0 05	<0 005		
		" 15 "	<1 1	<0 05	0 08		
		" 22 "	<1 1	0 5	0 08		
		" 28 "	1 5	1 0	0 04		
125	Control	Before	<1 1	<0 05	<0 005	Positive	Both positive‡
		After 3 days					

Agg = agglutinin

AT = International units of α antitoxin

AL = K units of antileucocidin

* Paralysed killed on 16th day

† Died on 7th day

‡ Died on 3rd day

The bacteraemia following intravenous infection

Quantitative blood cultures showed that staphylococci injected intravenously were rapidly removed from the circulation and that after one hour a very small proportion remained. Observations made on 16 immunised rabbits and 8 normal controls were analysed. There was no significant difference in the average counts one hour after infection. Twenty-four hours after infection the average counts were 155 for the immunised and 729 for the controls, the former being weighted with a count of 1200 in one rabbit. The difference between the 24-hour counts is 2.37 times the standard error of the difference.

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Titre of antibodies during the course of infection with Staphylococcus pyogenes and results of cultures from the tissues (experiment IV)

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		" 15 "	1 320	<0 05	0 005		
		" 22 "	1 160	0 1	0 08		
		" 28 "	1 80	1 0	0 01		
95	C	Before	1 80	<0 05	<0 005	Sterile	Both positive*
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		" 16 "					
109	B	Before	1 320	<0 05	<0 005	Sterile	One positive
		After 7 days	1 320	<0 05	<0 005		
		" 15 "	1 320	<0 05	0 16		
		" 22 "	1 160	6 0	0 16		
		" 28 "	1 40	3 0	0 16		
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		After 7 days	1 40	<0 05	<0 005		
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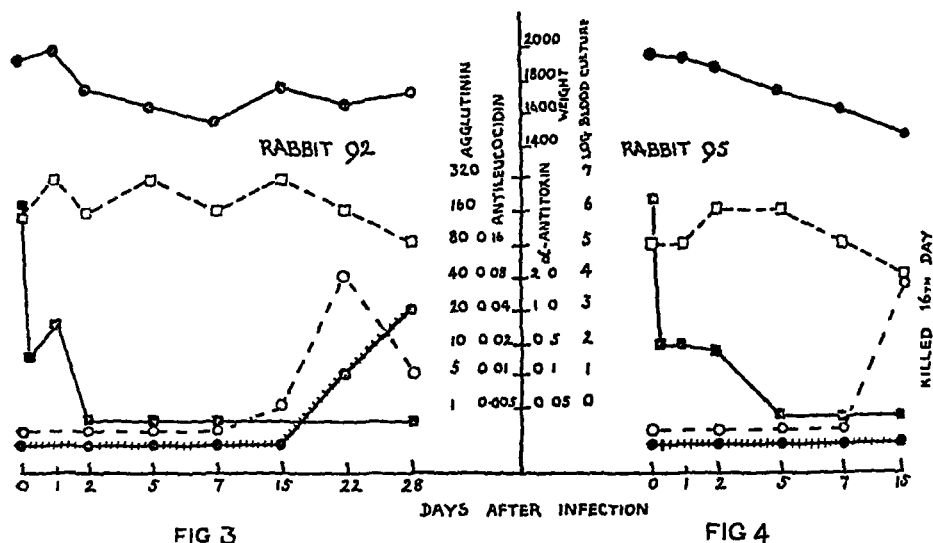
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FIGS 3 and 4—Changes in weight, bacteriæmia and serum antibodies during the course of infection

FIG 3—Immunised rabbit which survived infection

FIG 4—Immunised rabbit which became paralysed on the 16th day of infection

From these experiments it appears that immunisation did not enhance the normal clearing mechanism but possibly influenced the magnitude and duration of the secondary bacteriæmia

Sterilisation of the tissues

According to other workers, immediately after the initial clearance of the blood stream staphylococci are most numerous in the liver and spleen (Bartlett and Ozaki, 1916-17, Sullivan, Neckermann and Cannon, 1934, Forssman, 1937), but rapidly decrease in these organs and increase in number in the kidneys, where they may be present within an hour after infection (Dyke, 1923). In these experiments local abscesses have sometimes occurred in the myocardium, the costo-chondral junction and the bones, but if there were any abscesses the kidneys were always involved. Cultures taken from the pelvis of the kidney have been used as an index of the persistence of infection.

Staphylococci were recovered from both kidneys of all rabbits that died and from one or both kidneys of the majority that survived. Among the survivors the kidneys were sterile in 6 of 11 immunised and 1 of 2 normal animals.

Loss of body weight.

The weight of normal rabbits fell steadily during infection and those that survived for a fortnight had lost about 25 per cent

but with vaccine E, group 4 was not vaccinated Serum taken 8 days after the last dose of vaccine was tested for antibodies against

TABLE V

Results of intravenous infection with Staphylococcus pyogenes of specifically and non-specifically immunised rabbits

Group	Immunised with		No of rabbits	No surviving 28 days	Average survival time in days	Mean time to death in days	Percentage survived
	vaccine	No of doses					
1	D	9	11	4	20.5	16.1	47.1
2	D	3	6	4	21.7	9.0	
3	E	9	14	8	22.5	12.6	37.1
4		0	16	0	10.1	10.1	0

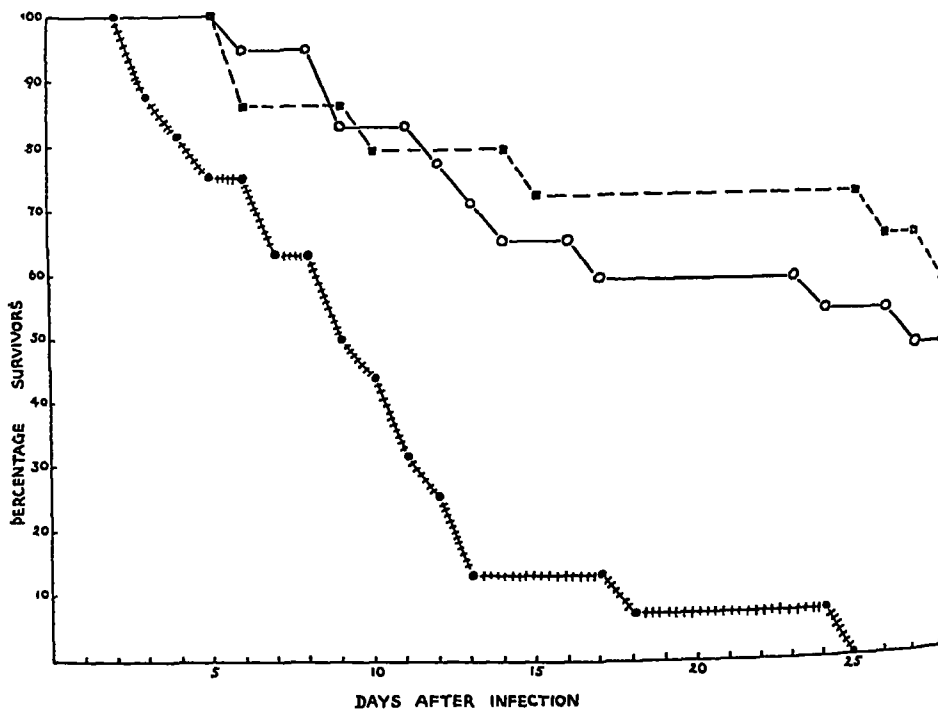


Fig 5—Time-survivor curves of immunised and non-immunised rabbits (experiment VII) Continuous line = groups 1 and 2, staphylococcal vaccine, interrupted line = group 3, pasteurella vaccine, barred line = group 4, normal controls

staphylococcus All the rabbits had <0.005 K antileucocidin and <0.05 unit α antitoxin per c.c. except a few in group 3 which had some α antitoxin before immunisation Agglutinin titres

less and lasted a shorter time than in the controls. All animals that had a secondary bacteraemia developed abscesses in the tissues and even after the blood was sterilised the majority of animals had foci of infection, the kidney being constantly affected. Complete sterilisation of the tissues was exceptional and it appears that the production of antibodies during infection was probably dependent upon the persistence of infection in the tissues. It is evident that the degree of immunity evoked by the staphylococcal vaccines employed was sufficient in most instances to sterilise the blood but not the tissues. This immunity did not depend upon α antitoxin or antileucocidin and was not clearly attributable to bacterial antibodies. It did however appear to involve a mechanism which tended to diminish the degree of secondary bacteraemia and to a certain extent it held the progress of the infection in check so that death was prevented or delayed. If animals survived long enough to produce antileucocidin their general condition frequently improved from about that time.

Whatever this mechanism may be it appears to be susceptible to non-specific enhancement. Vaccination with a pasteurella vaccine induced as high a grade of resistance as specific vaccination. This was not referable to staphylococcal antibodies for in this respect rabbits immunised with pasteurella and controls were alike.

The course of the bacteraemia was not studied in rabbits immunised with pasteurella but the similarity of the survival rate among the specifically and non-specifically immunised animals suggests that the increased resistance obtained with the staphylococcus vaccines was mainly non-specific. In so far as it operated against the bacterial cell it was antibacterial but there is no indication that it was specifically antibacterial. Possibly the experimental procedures may not have been optimal for demonstrating specific immunity.

SUMMARY

Rabbits immunised by intravenous inoculation of staphylococcus vaccine developed some resistance to intravenous infection with the same organism. A similar degree of resistance was produced by intravenous inoculation of a vaccine made from an antigenically unrelated bacterium. The development of antibodies in relation to the course of infection and the fate of the bacteria have been analysed.

I am very grateful to Dr F C O Valentine for his help in carrying out a large number of antileucocidin titrations.

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EXPERIMENTAL METHODS

The virus preparations employed were usually gradocol membrane filtrates prepared from infected tissues as described by Andrewes and Smith (1937) for influenza. The filtrates contained 50 per cent buffered broth and had a reaction of approximately pH 7.4-7.6. Dilutions were made in broth or saline. With vaccinia virus, the supernatant fluid from a well centrifuged 1 per cent emulsion of infected rabbit testis in saline was used.

Five per cent aqueous solutions of chemically pure bile salts were prepared for stock and sterilised by filtration through gradocol membranes. From these, solutions of the desired concentration were freshly made as required, in either distilled water or saline, the reactions always lay between pH 7.0 and 7.6.

Virus-bile salt mixtures were made by mixing equal quantities of dilutions of virus filtrate and bile salt solution. Control inocula invariably consisted of portions of the same virus dilutions mixed with distilled water or saline. Throughout the text and in the tables the stated concentrations of bile salt and virus refer to the final concentrations present in the mixtures.

The virus preparations were tested by the inoculation of susceptible animals by the appropriate route, thus influenza and louping-ill viruses were inoculated intranasally under ether anaesthesia into groups of young mice, vaccinia intradermally into rabbits, ectromelia intraperitoneally into mice and Rous sarcoma virus intramuscularly into young chicks.

The active bile salts, sodium deoxycholate and sodium apocholate, were found to be toxic for mice when given either intranasally or intraperitoneally, so that concentrations greater than 1/500 could not be injected. Even with 1/1000 dilutions, an occasional mouse died before recovery from anaesthesia. Therefore in experiments involving the use of bile salt solutions, in concentrations greater than 1/500 the preparation had to be diluted before test.

EXPERIMENTS WITH VARIOUS PATHOGENIC VIRUSES

Influenza virus.

Repeated experiments have shown that sodium deoxycholate and sodium apocholate rapidly destroy considerable amounts of influenza virus, the deoxycholate being somewhat more active. Sodium cholate has a slight viricidal action, sodium dehydrocholate none whatever. It is perhaps significant that the two active bile acids possess in high degree the capacity for forming stable additive compounds, and in one experiment it was shown that such an additive compound of sodium deoxycholate and stearic acid was totally devoid of viricidal power.

The amount of virus inactivated by bile salt was not directly proportional to the bile salt concentration. Thus a 1/500 dilution of sodium deoxycholate usually inactivated fifty times as much virus as a 1/1000 dilution.

Table I shows the results of an experiment in which three bile salts were tested in parallel.

Speed of virus inactivation. An experiment was designed to ascertain whether the viricidal action of bile salt was rapid or slow. Two sets of virus dilutions were made, sodium deoxycholate in a

final concentration of 1 1000 being incorporated in the dilutions of one set. The mixtures were tested at intervals as shown in table II. It is probable that the virus inactivation was practically instantaneous, for a 1 100 dilution of virus containing bile salt produced only a small lesion in one out of three mice, even when the inoculation was begun immediately after adding the bile salt, whilst the 1 10 dilution of virus produced extensive lesions in all three mice even 24 hours after the addition of the sodium deoxycholate.

Attempts to reactivate virus-bile salt mixtures. It is well known that the inactivation of many viruses by specific immune sera is a reversible phenomenon, at least for some considerable time after the reagents are mixed. It was shown by Andrewes (1928) that for vaccinia simple dilution of an inactive serum-virus mixture sufficed to restore infectivity. Attempts to demonstrate this dilution phenomenon with mixtures of influenza virus and sodium deoxycholate failed completely.

A further attempt to ascertain whether the virus inactivation was irreversible was made by washing the inactivated virus over a semipermeable gradocol membrane of pore size small enough to hold back influenza virus particles ($0.11\ \mu$). Ten c.c. of virus-bile salt mixture were placed in the container over the membrane and filtration under negative pressure allowed to proceed until 9 c.c. of filtrate had passed through. Nine c.c. of washing fluid consisting of 0.5 per cent Witte peptone in phosphate buffer at pH 8.0 were then added, and filtration continued until a further 9 c.c. of fluid had passed the membrane. This procedure was repeated 5 times, when the washed product lying above the membrane was collected, made up to the original volume and tested for infectivity. A control specimen of virus without bile salt subjected to similar manipulation had a titre of $1\ 10^4$. No reactivation of virus was obtained with the virus-bile salt mixture.

It is of course possible that a non-infective form of additive compound of virus and bile salt remained unaffected by the repeated washing, but in view of the experiments to be described with visible virus-like organisms, it is much more likely that the virus bodies are completely lysed by the sodium deoxycholate and thus irreversibly inactivated.

Louping-ill virus

The virus of louping ill is as sensitive to the destructive action of sodium deoxycholate as is influenza virus. A 1 1000 dilution of the bile salt has been repeatedly shown to inactivate completely a virus filtrate with a titre of $1\ 10^3$ or $1\ 10^4$, whilst a 1 2000 dilution has produced partial inactivation.

This virus, however, is relatively unstable and the potency of a

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TABLE IV
*Showing the effect of different concentrations of three bile salts
upon suspensions of sewage organism A*

Density of organism suspension equal to	Bile salt	1:1 ml dilution of bile salt											Saline controls
		1 1000	1 2000	1 3000	1 4000	1 5000	1 6000	1 7000	1 8000	1 9000	1 10,000	1 20,000	
2000×10^6 <i>B coli</i> per c c	Sod deoxycholate	-	tr	±	+	+	+	+	++	++	++	++	++
	Sod apocholate	+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	Sod cholate	+++	+++	+++	+++	+++	+++	+++	+	+++	+++	+++	+++
200×10^6 <i>B coli</i> per c c	Sod deoxycholate	-	-	-	-	-	-	-	tr	tr	tr	+	+
	Sod apocholate	-	-	-	tr	+	+	+	+++	+++	+++	+++	+++
	Sod cholate	-	±	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

-- = clear tube.

tr, ±, +, etc = increasing degrees of opacity

accepted that the bile solubility of pneumococci is merely the acceleration of the autolysis which occurs in old' cultures (Mair, 1929), brought about by an autolytic ferment of the bacterial cell. If pneumococci are heated to 56°C for 30 minutes they become completely resistant to the action of bile salt, presumably because the autolysin is thermolabile and has been completely destroyed. If such heated organisms are resuspended in an unheated culture, they regain their bile solubility.

It is possible that virus inactivation by bile salts is also dependent upon the presence of autolytic ferment, and it may be significant that those viruses which are susceptible decline in infectivity more quickly when kept either in cold storage or at 37°C than the bile-resistant viruses of vaccinia and ectromelia. The visible organisms of pleuropneumonia and sewage, however, do not behave like pneumococci in certain respects. I have never seen spontaneous lysis in cultures even when kept for some time after all the organisms had died. Also the ageing of cultures, instead of increasing bile solubility as in the case of pneumococci, decreases it so that it may be impossible to obtain complete clearing of an old culture even with a 1:100 concentration of sodium deoxycholate. This is due to some change in the organisms themselves, not in the medium, because washed suspensions likewise exhibit bile resistance. The old cultures or suspensions prepared therefrom show a slight diminution of opacity on the addition of bile salt and it was thought at first that this might be due to the development of bile-resistant mutants on long incubation so that the culture consisted of a mixture of bile-soluble and bile-resistant units. That this interpretation is incorrect is shown by the fact that young cultures, heated to 56°C for 30 minutes, exhibit the same phenomenon, the addition of 1:1000 sodium deoxycholate causes a slight diminution of opacity, but the addition of further bile salt up to a concentration of 1:100 produces no further clearing. The reason for this slight clearing effect is unknown.

An experiment was made to see whether living bile-susceptible organisms could transmit their susceptibility to bile-resistant heated organisms. A Fildes' broth culture of sewage organism A was divided into two parts, one being heated at 56°C for 30 minutes, the other left untreated. Washed suspensions in saline were prepared from each. The addition of one-tenth volume of 1 per cent sodium deoxycholate to samples caused slight diminution of the opacity of the heat-killed suspension, and complete clearing of the unheated suspension, although this latter was purposely made considerably denser than the former. Heated suspension was now mixed with an equal volume of original culture and the mixture left for 1 hour. The addition of bile salt resulted in a product which had exactly the same opacity as a control mixture of heated

IMMUNOLOGICAL EXPERIMENTS WITH BILE-INACTIVATED VIRUSES

The most obvious practical application of the phenomenon of bile solubility of viruses is the use of lysed products for immunological investigations. So far very few investigations of this kind have been made and none have yielded encouraging results.

An antigen was made by grinding influenzal mouse lungs in a 1:1000 dilution of sodium deoxycholate to make a 5 per cent suspension of the tissue. This was first clarified by filtration through asbestos pulp and then passed through a 0.79μ gradocol membrane. The final product was not completely inactivated—it contained roughly one hundredth as much living virus as the stock influenza virus filtrates prepared in the same way but without the addition of bile salt. It was used to immunise a group of mice by giving one subcutaneous dose of 0.25 c.c. and an intraperitoneal dose of 0.5 c.c. 2 weeks later. The mice when tested later by intranasal instillation of virus under ether anaesthesia showed the same degree of resistance as would have been expected if a 1:100 dilution of untreated virus filtrate had been employed as antigen. When the bile salts were removed by precipitation at pH 5.0, the resulting supernatant fluid proved to be a still worse immunising agent. The toxicity of the sodium deoxycholate makes its removal desirable from any preparation intended as a vaccine, and possibly precipitation with acid, in which a bulky gelatinous precipitate forms, is unsatisfactory because of the large adsorbing surface produced. It is intended to investigate the activity of products in which bile salt has been removed by dialysis.

Similar lysed preparations have been used in attempts to demonstrate a precipitation reaction with specific immune sera. Both ferret and mouse influenza lung filtrates have been employed. When mixtures of the antigen and antiserum have been incubated in a water-bath at 37°C no specific precipitation has occurred, even after 24 hours' contact, but with ring tests in which the lysed antigens were layered over the surface of sera, some slight evidence of specific reaction was obtained. Both immune and normal sera gave distinctly visible rings at the interface, but those with immune sera were very much more pronounced and appeared very much more rapidly. Attempts are now being made to prepare stronger antigens by grinding infected tissues in much more concentrated solutions of bile salt and then removing the latter either by acid precipitation or by dialysis.

COMMENTARY

The work outlined above has shown that some viruses are readily inactivated by certain bile salts, probably with lysis of virus bodies, whilst other viruses remain completely unaffected.

3. The susceptibility of a virus to bile salts is not related to its size.

4 The process of bile salt lysis can be observed both macroscopically and microscopically with the cultivable virus-like organism of pleuropneumonia and the sewage organisms of Laidlaw and Elford

5 Attempts to use lysed virus preparations for prophylactic immunisation have so far not been encouraging

I am greatly indebted to Dr O Rosenheim, who supplied all the bile salt preparations used throughout this investigation

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and (c) 10 c.c. of nutrient agar at 50° C which was poured into a plate. When dealing with anaerobic streptococci, liver broth containing liquoid and, as a control, liver broth containing 5 per cent. trypsin were used. The amount of growth was estimated by the opacity after 24 hours' incubation (48 and 96 hours with anaerobic streptococci) and at the same time subcultures to blood agar plates were made to verify the result and to exclude contamination.

Abolition of the bactericidal power of the blood. The method previously described in detail (Hoare, 1938) for bactericidal tests on the blood was used.

Discussion

Table I shows the results obtained with hæmolytic streptococci (groups A, B, C and G), *Bact. coli* and *Staphylococcus aureus*. The liquoid prevented the formation of any blood clot, and satisfactory growth was obtained from small numbers of implanted organisms.

TABLE I
Effect of liquoid in culture media on the growth of various organisms from artificially infected blood

Test organism	Number of bacteria inoculated	Growth in glucose broth + liquoid	Growth in liver broth + liquoid	Liquoid (per cent.)
Hæmolytic streptococcus group A	36 6	++++ +++	++++ +++	0.03
Hæmolytic streptococcus group B	72 6	++++ ++++	++++ ++++	0.03
Hæmolytic streptococcus group C	20 3	+++ ± ++ 48 hrs	+++ ++	0.03
<i>Bact. coli</i>	100 12	+++ +++	+++ +++	0.03
Hæmolytic streptococcus group A	70 9	++++ ++++	++++ ++++	0.2
Hæmolytic streptococcus group B	20 2	++++ — + 48 hrs	++++ — + 48 hrs	0.2
Hæmolytic streptococcus group C	30 4	++++ ++++	++++ ++++	0.2
Hæmolytic streptococcus group G	32 5	++++ ++++	++++ ++++	0.2
<i>Bact. coli</i>	87 10	++++ ++++	++++ ++++	0.2
<i>Staphylococcus aureus</i>	81 11	+++ +++	++ ++	0.2

± to ++++ = varying amount of growth after 24 hours' incubation

These results show that liquoid, although quite suitable for most organisms, is unsuitable for use in culture media for the isolation of anaerobic streptococci from the blood. Unless suitable methods of anaerobic cultivation are employed the presence of anaerobic streptococci will frequently be overlooked. In addition to being isolated from the blood in puerperal septicaemia (Schottmuller, 1910, 1924, Bingold, 1921, Lehmann, 1926, Schwarz and Dieckmann, 1926, 1927, Colebrook, 1930; Brown, 1930) anaerobic streptococci have been found in the blood in cases of putrid empyema (Fisher and Abernethy, 1934). Apart from lesions such as lung abscess, empyema, endocarditis and peritonitis secondary to puerperal pelvic infection, anaerobic streptococci have been found in various gangrenous lesions in the lungs and pleura (Davis and Pilot, 1922, Lambert and Miller, 1924, Eggers, 1926; Cohen, 1932, Fisher and Abernethy, 1934), in infection of the urinary tract, otitis media and mastoiditis (Rist, 1905), in tonsillar and alveolar abscesses (Lehmann) and in hepatic abscess (Fisher and Abernethy). In certain of these reported cases true obligatory anaerobic streptococci were isolated, but in others the strains were anaerobic by predilection only. Suitable medium for the isolation of anaerobic streptococci from the blood is either Martin's meat medium under a paraffin seal, or a liver digest broth (Colebrook and Hare, 1933) containing 5 per cent trypsin.

Conclusions

Liquoid, like trypsin, abolishes the bactericidal power of normal blood and does not interfere with the cultivation of most of the common pathogenic bacteria, even when these are present in very small numbers. The presence of liquoid, however, was definitely unfavourable to the growth of anaerobic streptococci (5 strains), and since these organisms are frequently present in the blood in puerperal fever—and less frequently in other conditions—the routine employment of liquoid for blood cultures is not to be recommended.

Sterile trypsin, prepared by Messrs Fairchild and now being supplied again by Messrs Burroughs Wellcome under the name "Solution of trypsin," has no such inhibitory effect.

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such localisation about the infected paw is exceptional. In most cases the disease becomes progressive, with "metastasis" to the tibio-tarsal joint of the other hind paw, which begins to swell, until eventually both paws may attain double or three times the normal diameter (figs 1-4). In the joints themselves there is at this stage a greater or lesser quantity of fluid which is usually bacteriologically sterile, but which, nevertheless, is very infective. Occasionally suppuration has occurred, but in such cases it may be inferred that there has been secondary infection. When this condition prevails the polyarthritic process usually ceases. A number of the animals die, whilst others slowly recover, even though the swollen parts may not return to the normal size. In some rats the carpo-metacarpal, metacarpo-phalangeal and even the interphalangeal joints of the two front legs also become affected, and in very rare instances a thickening of the caudal vertebræ is observed. The coat becomes rough, and the rat moves about rather clumsily on account of its deformed paws. Microscopically no changes are seen in the internal organs. Occasionally one may find a small quantity of pericardial fluid or a quantity (up to 1.5 c.c.) of watery exudate in the pleural sacs, containing very few cells. Even more rarely there is some peritoneal exudate. In none of these exudates, however, are bacteria to be found, microscopically or culturally (see below). The changes developing in the joints and spreading into the bony substance are illustrated in the accompanying X-ray photographs taken through the courtesy of Professor van der Plaats (figs 2 and 3).

The disease may be transmitted by various routes as shown in table I. Thus it will be seen that out of 277 rats injected

TABLE I.
Susceptibility of white rats to infectious polyarthritis

Mode of infection	Number inoculated	+	-
Plantar	277	270	7
Scarification	15	15	0
Subcutaneous	8	5	3
Intraperitoneal	9	4	5

subcutaneously in the planta, 270 contracted the disease. Similar results are obtained when infectious material is rubbed into the scarified paw. After intraperitoneal injection of 9 rats, 4 contracted the disease and showed, besides the usual local polyarthritis, changes in the distal extremities and very marked pericardial and pleural exudation. Healthy rats, even though scarified, kept for a period of months in the same cages as diseased rats did not become infected.

(1931) The former claims that the ætiological agent of arthritis in mice is a coccobacillus, while the latter investigators have isolated a corynebacterium which they regard as the cause. The results of our own experiments may be given here in some detail.

Rat 219 c Plantar infection induced on 2nd April 1938. On 11th May both hind and fore legs showed severe swelling. Heart blood, pleural exudate, liver, spleen, brain and synovial fluid from left fore paw and left hind paw were inoculated on ascitic agar, Löffler's serum and blood agar. The material from each source was streaked separately on two plates, one of which was cultivated aerobically, the other anaerobically. The anaerobic plates remained entirely negative, as did the aerobic plates of ascitic agar and Löffler's serum. On the blood agar plates inoculated with heart blood minute colonies of non-hæmolytic streptococci grew.

Parallel with the bacteriological tests a portion of the same material was inoculated also into the plantar region of three healthy rats in each instance. In this experiment it was found that the rats injected with blood and brain substance remained healthy, whereas those injected with pleural exudate, liver, spleen and material from the joints contracted the disease.

Rat 201 a. Injected in the plantar region on 25th April. Forty-four days later both hind paws were greatly swollen and the two front paws to a less extent. Heart blood, pleural exudate, liver, left hind and front paws were cultured aerobically on blood agar, ascitic agar and Löffler plates. Except for some contaminants no growth was observed aerobically in five days. Rats injected with material from the joints and blood became infected but not those inoculated with material from the liver or with the pleural exudate.

Rat 189 a Injected in the plantar region on 25th April. On 8th June both hind paws and one front paw had become greatly swollen, the remaining front paw less so. Heart blood, pleural exudate, liver, right front and left hind paws were streaked on blood agar, ascitic agar and Löffler plates and inoculated aerobically. Except for a few contaminants the results were negative. All the material except the blood produced infection.

Rat 219 d Injected in the plantar region on 2nd April. On 8th June all four paws were much swollen. Heart blood, pleural exudate, liver, right hind paw and a pus-like liquid from the right front paw were streaked on plates of blood agar, ascitic agar and Löffler. All plates remained sterile except that from the hind paw, where non-hæmolytic streptococci developed. All material inoculated into rats in the planta caused polyarthritis.

Rat 219 e Injected in the planta on 2nd April. On 8th June all four paws were greatly swollen. Heart blood, pleural exudate, liver, right hind paw and left front paw inoculated as above. All plates, except for a Löffler plate with contaminants, remained sterile. All the material inoculated into rats produced symptoms of polyarthritis.

Summarising our experience with these rats it may be stated that in no instance was it possible to cultivate a micro-organism that might be considered specific for the disease. Except for the plates with the non-hæmolytic streptococci only a few contaminants were observed and these were clearly recognised as such. In contrast the same material was usually distinctly pathogenic for rats when injected.

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It must be particularly emphasised that the agent of polyarthritis in rats exhibits a very distinct tropism towards the joints, and this is to be observed also with subcutaneous, pulmonary, intraperitoneal and other methods of infection. In *C. arthritis muris* we have a micro-organism which is pathogenic for various rodents but which produces abscesses and pus only when inoculated into the joint. Other corynebacteria pathogenic for rodents have previously been described by Levy and Fickler (1900), Klein (1902-03) and Bergéy (1904).

The presence of the agent in the organs.

To determine the distribution of the causative agent of polyarthritis of rats portions of the organs of infected animals were ground in mortars and inoculated into white rats and field rats.

In table III the second column indicates the duration of the infection in the rat from which the various organs and fluids were taken and the third the clinical picture in this rat as it was on the day of inoculation. It will be seen that the inoculation of joint material into white rats was positive in all 12 cases. In 6 the agent could be demonstrated in the blood, in 2 in the brain, in 4 in the lungs, in 3 in the pleural exudate and in 5 in the liver. In one instance the peritoneal exudate gave a positive result.

TABLE III

Transference of infection to white rats with organ emulsions and body fluids of infected white rats

No	Interval since infection (days)	State of infected rat from which organs were obtained	Results of injection of						
			joint extract	blood	brain	lungs	pleural exudate	liver	peritoneal exudate
1	31	Both hind paws positive		+	-	-		+	
2	40	All paws positive	+	-	-		+	+	
3	45	" "	+	+			-	+	
4	45	" "	+	-		+		+	
5	47	" "	+	+	+		+	+	
6	47	Both hind paws positive	+	+	-		+		+
7	47	" " "	+	+		-		-	
8	48	" " "	+	-	-	+		-	
9	48	" " "	+	+	-	+		-	
10	48	One hind paw positive	+	-	-	+		-	
11	48	" " "	+	-	+	+		+	
12	48	Became negative	+	-	-	-		-	

The organs of 8 infected white rats were also inoculated into field rats, with similar results except that in one instance the inoculation of the substance of the diseased joints and of other organs failed to cause infection (table IV).

These experiments indicate that in the polyarthritis of rats the agent is not confined to the diseased joints. It is to be found in the blood, lungs, pleural exudate, liver, spleen and peritoneal exudate. In the brain it has been found rarely, in one instance it was present there though absent from the blood. The blood itself

whether the agent persisted in the reinfected rats and, on the other, the small number of animals available made it impossible to carry out quantitative experiments. Consequently the question must remain undecided whether we are dealing here with a reinfection or with a super-infection.

A somewhat larger number of white rats were at our disposal in which the disease had been confined to one hind leg or in which at least one of the hind paws, after having swelled up, had resumed its normal aspect and could be used for reinfection. Here, therefore, we had to deal with rats still exhibiting evidence of the disease, but it remains an open question whether the host still contained the infecting agent or whether the symptoms in the joints simply persisted. In all, 59 such rats were reinoculated, the interval between injections varying from 35 to 124 days. Only 3 rats reacted positively, the remaining 56 proved to be immune.

Furthermore there were 116 field rats from different series in which no symptoms followed the first inoculation, although other rats of the corresponding series contracted the disease. The results are shown in table V.

TABLE V

Result of the reinoculation of field rats which remained negative after the first inoculation (latent infection)

Mode of infection	Interval (days)	Result of reinoculation		
		No reinoculated	+	-
Scarification	35	2	1	1
"	49	14	14	0
Plantar	26	1	0	1
"	36	3	2	1
"	37	5	1	4
"	43	4	0	4
"	45	11	7	4
Subcutaneous	35	20	10	10
"	49	15	9	6
Intraperitoneal	35	24	2	22
"	49	7	0	7
Pleural	35	10	0	10

If one does not take into consideration the results obtained with the scarified rats, reinfection of which succeeded in 15 out of 16, it is found that in all the other series there is a very considerable percentage of immune individuals. Out of 24 which had been inoculated previously by the plantar route 14 remained immune, out of 35 previously inoculated subcutaneously 16 remained

father and son, aged 53 and 19. Almost no normal dentine was found, there being a great lack of Tomes' fibrils, the pulp was replaced by pulp-stones and there were very numerous structural defects which are clearly the same as the "canals" of Naito. He noted also the ease with which the enamel became separated from the dentine.

Bergersen (1929-30) examined the mandible of a child who died in his third year. The incisors were small but of good structure, especially in the outer layers of the dentine, they showed minor changes in the pulp and odontoblast layer and the inclusion of cells in the normally cell-free cementum. The first deciduous molar was more disturbed, showing patchy staining, lamination of the dentine and unequal calcification, but Tomes' fibrils were present in almost normal numbers. The canine had a thin good outer layer but otherwise was very unequally calcified and contained but few Tomes' fibrils. The odontoblasts were least affected towards the root end. The pulp resembled ordinary connective tissue. He drew attention to the resemblance to the teeth of scorbutic animals. In the permanent lateral incisor and canine tooth germs he observed degenerative changes in the enamel organ.

Becks (1931) reported on the condition of the teeth of a full-term foetus described by Weber in 1930. He found that in an incisor tooth the dentine was sharply divided into two layers. The outer layer contained Tomes' fibrils and was less defective than the inner, which resembled the tissue described by Bauer. Von Korff's fibres were not formed except in the younger parts at the root end of the germ, and only there were almost normal odontoblasts with Tomes' fibrils present. The dentine of the canine and molar germs resembled the outer layer in the incisor.

Pfuger (1932) referred to a tooth germ in which the peripheral dentine only was almost normal.

Fleming, Radasch and Williams (1937) examined a tooth from a child. The dentinal tubules were irregular in course, shape, and distribution.

The frequency of macroscopic dental defects in osteogenesis imperfecta is not known, since even in the limited number of cases in which the condition of the teeth has been recorded, it is not clear whether the record refers to all teeth or to which. Where they have not seemed normal the terms applied vary from brown or grey-brown (Preiswerk, 1912, p. 46, first description) to yellow and translucent or even transparent (Sprawson, 1931). In Adloff's cases the permanent teeth were deficient in enamel and brown (though not decayed) as regards the crowns, but the roots were translucent. Thoma (1936) has noted that the roots are usually short. Mr H. A. T. Fairbank, who has examined some thirty cases, informs me that judging by naked-eye appearances the permanent teeth are commonly less affected than the deciduous, even in so-called post-natal cases.

MATERIAL

The teeth studied were an upper permanent central incisor tooth, two upper molars thought to be the second, and an upper third molar. They were removed from a dwarfed crippled woman about 30 years of age who attended as an out-patient at the dental department of Guy's Hospital. The teeth were preserved for me on account of their peculiar pinkish colour, and as the patient could not afterwards be traced it was impossible to obtain details of her history beyond the bare facts that she had had numerous fractures of the bones and had been treated for years as a case of

are present at the periphery of the whole tooth, though differing little in refractive index from the matrix and thus remaining scarcely visible. The deep layers of the dentine contain large numbers of cells with coarse irregular canaliculi or are hyaline. The dentine also contains many concentrically laminated pulp stones (fig 1). There were no typical interglobular spaces to be seen.

Decalcified sections

The teeth became decalcified more quickly than normal teeth.

1 The anterior upper molar.

The Tomes' fibrils The Tomes' fibrils at the extreme periphery of the dentine alone appear normal (fig 3), and this in all parts of the tooth, so that whatever factor caused the abnormality of the rest of the dentine was related rather to the age of the odontoblasts than to the age of the patient. The layer containing normal Tomes' fibrils is wider in the roots than in the crown, where it is in places extremely thin.

Central to the zone of normal dentine lies a zone where the tubules are interrupted by areas without tubules. These areas have a sharp border, convex towards the outside of the tooth (fig 3), and at their middle an uncalcified or partly calcified structure extending in the direction of the pulp (the "canals"). More central still is a zone in which these structures become more numerous and normal tubules are scarce. The original tubules do not reach the neighbourhood of the pulp, most of which has been replaced by calcified tissue containing in parts great numbers of cells with irregular tubular processes, but a little pulp tissue remains in the roots. Incorporated in the dentine are many large and small spherical calcified masses, some of which contain tubules at their centre, but which are otherwise without tubules and are concentrically laminated. They represent free formations arising in the pulp of the growing tooth and afterwards surrounded by the advancing dentine. There is an abnormal variation in the size of the tubules, and one or two end in a bulbous expansion in the middle of the dentine which probably represents an included odontoblast.

The dentinal matrix The matrix at the extreme periphery of the dentine does not appear abnormal, though this layer is very thin in parts on the top of the crown. In the immediate neighbourhood of the commencement of the "canals" it stains a homogeneous yellowish pink or red (similar to pre-dentine) in hæmatoxylin and eosin or van Gieson preparations, indicating poorly calcified collagen. This relationship is maintained throughout the course of these structures towards the pulp, so that in a cross section

the more normal dentine at the periphery. In the rest of the dentine the usual ring markings are very rare and interglobular spaces absent. The depth of staining with hæmatoxylin is no reliable guide to the degree of calcification in these specimens, since this depth is affected also by the collagen content.

The "canals" Examination of the peripheral end (or rather beginning) of the "canals" shows that, at the time they started, the formation of a group of Tomes' fibrils ceased abruptly and that the matrix in this region terminated in a curved border equally suddenly but somewhat proximally (fig. 7). The direction of lamination of the surrounding dentinal matrix shows also that matrix formation lagged in this neighbourhood (fig. 5).

In ground sections the commencement of the "canal" often appears as a clear tubular loop, the shaft more frequently as a connected series of irregular elements arranged in a column (fig. 2). The contents have undoubtedly become partly calcified, and though judging by hæmatoxylin staining the calcification seems poor it does not appear so in a ground section. In hæmatoxylin and eosin-stained sections the "canals" appear, where cut transversely, as wide faint blue tubes, either empty or containing smaller hollow elements or unrecognisable material. In longitudinal section their commencement often contains a large tubular loop convex towards the periphery of the dentine (fig. 8), and they usually include portions of large tubes in various parts of their course. The contained tissue has, however, been cut off from all sources of nourishment for a long time and it stains in a ghostly manner. The appearance in these sections strongly suggests that the "canals" formerly contained capillary loops.

Cross sections of the "canals" stained by Orban's method appear as clear circles surrounded by almost homogeneous collagenous matrix and containing a group of transversely cut coarse collagen fibres arranged just within the limit of the "canal," like a stockade or irregularly. In longitudinal sections these same fibres are seen, sometimes of great length, with portions of wide tubes among them (fig. 5).

The pulp The remnants of the pulp are very small. There is no odontoblast layer or normal pre-dentine zone, but in their place are numerous large collagen bundles arranged at right angles to the inner surface of the dentine, and fibres from these can be traced into the calcified tissue and pulp (fig. 9). The arrangement is, of course, not peculiar to this disease, and resembles that found where cementum is being laid down. In addition to the collagen bundles there is a mesh of finer argyrophil fibres, each, however, much thicker than normal von Korr's fibres. From these various bundles, no doubt, was formed the matrix of the calcified tissue which occupies most of the former pulp cavity. The pulp contains

PLATE LXVIII

- FIG. 4**—Decalcified section of root of anterior molar. The Tomes' fibrils at the periphery are cut obliquely, those few in the centre transversely. Numerous vascular "canals" are seen in cross section surrounded by pale zones of collagenous matrix which are separated by dark areas lacking in collagen. A thick covering of cementum is shown. Picro-thionin $\times 60$.
- FIG. 5**—From a section of the same molar showing the dentinal matrix just beneath the good dentine. The peripheral end of a vascular "canal" is in the centre and portions of others are to left and right. These are surrounded by collagenous matrix (uniformly dark) and contain coarse longitudinal collagen fibres and large tubular elements. The rest of the matrix is largely argyrophil and in parts (light) lacking in fibres of any kind. A horizontal line of lamination shows a festooning effect indicating retarded matrix formation in the neighbourhood of the included capillary. The Tomes' fibrils are unstained and may be seen here and there as very fine vertical white lines. A section of a normal tooth stained by this method would show them in great numbers on a uniform dark ground. Orban's stain $\times 160$.
- FIG. 6**—Section from middle of crown of same tooth, showing the tangential arrangement of argyrophil fibres about the unstained transversely cut dentinal tubules, similar to the normal arrangement in pre-dentine. The matrix at the upper right corner is collagenous. Orban's stain $\times 750$.

in experimental rickets. But in both rickets and tetania parathyroopriva the dentine is not lacking in Tomes' fibrils or collagen.

The occasional occurrence of isolated capillaries in the dentine of man is well recognised, but such an extensive and relatively orderly vascularity as that here described has, I believe, only once been recorded, namely by John Tomes (1848), as Mrs Lindsay has kindly pointed out to me. He wrote "Recently I have met with a specimen in which the dentine, both of the crown and fangs of a molar tooth of the lower jaw, is traversed by numerous canals, which form loops directed either towards the coronal surface, or that of the fangs.

"These are evidently, both from their size and arrangement, the vessel of the pulp calcified. They are obliterated at parts, and do not look as though blood had recently circulated through them."

It has been shown that in my specimens there is evidence that a whole group of odontoblasts immediately distal to the capillary loop ceased to function shortly before the loop began to be included in the dentinal matrix, and it seems certain that a determining factor in the inclusion of the vessels is cessation or retardation of matrix formation at one point compared with the rate of formation in the neighbourhood. Various studies, such as those of Westin on human scurvy and of Pohto (1938) on vitamin A deficiency in rats, support this view. This consideration may explain the rarity of vascular dentine in foetal cases of osteogenesis imperfecta, where usually all odontoblasts of the same age are equally impaired.

Increased vascularity of the pulp, which has been commonly observed in the foetal cases, may determine the vascular nature of the included tissue.

The odontoblasts and Tomes' fibrils

It is clear that in many cases of the disease where the teeth are severely affected the odontoblasts are normally differentiated, maintain at first their epithelium-like order and produce for a while normal Tomes' fibrils, and this at a time when older odontoblasts are already suffering extreme disability. As they grow older the odontoblasts become incapacitated, either gradually and a few at a time as described above, or all of about the same age together as in many of the foetal cases. Similar events occur in scurvy and hereditary opalescent dentine.

Scurvy In both osteogenesis imperfecta and scurvy the formation of Tomes' fibrils stops, matrix formation continues except in "complete scurvy" (Wolbach and Howe, 1926) and is defective in collagen (Hojer and Westin, 1924), pulp-stones are formed and the older odontoblasts at the crown end of the forming tooth are affected before the younger ones at the root end.

PLATE LXIX

- FIG 7 —Section from crown of same tooth. The upper part shows the more normal dentine at the periphery, the surface of which has been worn away during life. The Tomes' fibrils (dark) cease abruptly at a sharp line, or continue, diminishing in number and becoming very scarce near the bottom of the figure. In the centre is the commencement of a vascular "canal" separated by a sharp boundary from the surrounding collagenous matrix (light). The dark streaks within it are not Tomes' fibrils. The ill defined dark areas to left and right and the spidery markings are regions where the matrix is lacking in collagen. Picrothionin $\times 160$
- FIG 8 —Section from same tooth, showing tubular loop at the peripheral end of a vascular "canal". The lighter, trunk-like area at the bottom left corner is a zone lacking in collagen and accidentally defined by slight crumpling. Hæmatoxylin and eosin $\times 340$
- FIG 9 —Periphery of pulp in anterior molar root, showing arrangement of collagen bundles perpendicular to pulp wall (right), dense masses of collagen in the pulp (left), and the distribution of collagen fibres in the recently formed calcified tissue. Orban's stain $\times 180$

of a foetal case a normal amount of intercellular substance was only present around the blood vessels offers some corroboration. It may also have been able to become collagenous because it remained long uncalcified, for it is a common observation that where capillaries are included in human dentine the surrounding matrix does not calcify to any great extent or only after much delay.

The absorption of collagen around the pulp of the third molar
The fact that the carious process in the crown of the tooth had reached the coronal part of the pulp cavity makes it impossible to say with certainty that the absorption of the hard tissue around the walls of the pulp cavity and within it was unconnected with the infection. This may, however, have been the case since a similar absorption was found by Westin in cases of early healing scurvy, and it is possible that here also it may represent an early healing change.

Cementing substance

Another component of normal dentinal matrix which must be mentioned is the so-called cementing substance. This has not been clearly defined but is understood to be that substance which lies between the fibrils of the matrix, which renders invisible the primitive fibrillar structure of the matrix and in connection with which the calcium salts are normally deposited.

In the sections here described the argyrophil fibres particularly, and in many parts the collagen fibres also, lie in a clear substance which is unaffected by any of the constituents of Orban's stain except sometimes Azur II. In a normal tooth this material, if present, is completely masked by other stainable constituents, and the fibrillar structure can only be demonstrated in the odontogenetic zone by the same method. A gross deficiency of cementing substance is therefore probable though deficiency of collagen may also be a cause.

Calcification

The softness of the teeth on grinding and the rapidity with which they became decalcified show that calcification was insufficient, though the tendency of the teeth to split to lose their enamel and to break up without causes in the mouth may be due to the deficiency of collagen. But the translucent appearance, with absence of the granular layer of Tomes' and obliteration of dentinal tubules, suggests that a considerable degree of secondary calcification occurred.

Chronology

The fact that some investigators found no changes in teeth from cases of osteogenesis imperfecta while others did so might be explained on the ground that only those teeth or parts of teeth

PLATE LXX

- FIG 10 —Pulp remnant in same tooth as fig 9, showing diffuse calcification of pulp and abnormal calcified tissue about it. Hæmatoxylin and eosin $\times 40$
- FIG 11 —Section of root of third molar. Peripherally are thick layers of cementum, replacing in some parts tissue previously absorbed. Proceeding towards the pulp, a layer of good dentine is next reached, then abnormal dentine containing radiating capillaries each surrounded by dark-staining collagen. Next, in the lower half, comes a line which defines the limit of previous absorption of dentine from the pulp side. The absorbed dentine has been replaced by further calcified tissue (very dark), and this in turn has now been partly absorbed all along the pulp side. Orban's stain $\times 12$
- FIG 12 —Third molar root, showing evidence of extensive absorption of the pulp-wall and of calcified tissue within the pulp. The porous character of the last-formed "dentine" can also be seen. Van Gieson $\times 60$

Tomes' fibrils were produced at the periphery of the teeth but soon the odontoblasts and other cells concerned ceased to form Tomes' fibrils and normal matrix. This change occurred first where the odontoblasts were oldest, but in a piecemeal manner. Focal defects in the rate of formation of the matrix led to the inclusion therein of blood vessels. The peripheral pulp cells produced pre-collagenous argyrophil fibres but these were not converted into collagen except in the immediate proximity of blood vessels. Pulp stones of good collagen content occurred in the middle of the dentinal papilla. The matrix was inadequately calcified and probably lacking in cementing substance.

At a later stage, when no normal Tomes' fibrils were being formed, a tissue resembling primitive fibre bone replaced the former pulp cavity. Most of the dentinal tubules which had been formed became occluded.

I am indebted to Professor B. Bergersen of the Tannlaegehoeiskole, Oslo, for the opportunity of examining the sections from his case of osteogenesis imperfecta, to Dozent Dr Gosta Westin of Karolinska Institutets, Stockholm, for allowing me to study his human scorbutic material, to Dr I. Schour of the University of Illinois for the gift of sections, to Dr Evelyn Sprawson for the loan of specimens, and to Mr J. F. Dudley, who has made all the microscopical preparations and photographs.

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Any freshly drawn exudate may be used, if, as in the experiment recorded here, fluid from a burn blister is used, the burn must have occurred at least 24 hours before. As a control fluid any transudate, normal serum or saline may be used, broth is unsatisfactory as it may contain a similar toxic substance liberated during the incomplete digestion of protein by trypsin.

The results are set out in the table. The survival time in the experimental animals was reduced to half that in the controls. One of the control animals died of shock following the injection and one other control was killed for histological examination on the day on which the last experimental animal died. This experiment has been confirmed with other groups of animals and with other samples of exudate, including the clinically relevant "simple pleural effusion."

Morbid anatomy and histology

The morbid anatomy and histology of the two groups confirmed the differences between them.

The spleen, liver and lungs were constantly affected. Involvement of other organs was variable.

The individual lesions in the control group were small submiliary tubercles. Microscopically there was good fibrosis, little if any necrosis, a good cellular reaction of healthy mononuclear cells and lymphocytes, an occasional polymorphonuclear cell and many well formed giant-cell systems (fig. 1). Not more than one or two bacilli were seen in a microscopic field (1/12 in objective). In most of the sections no bacilli were seen.

In the experimental group the appearance was that of much more active disease. The lesions were larger (though not noticeably more numerous) and necrotic and tended to be confluent. Microscopically there was scarcely any fibrosis, necrosis was marked, the cellular reaction, such as it was, contained many more polymorphonuclear cells, most of the mononuclear cells were degenerate and giant-cell systems were either absent or very poorly formed. Tubercle bacilli were abundant in all fields, and in some organs, notably the lungs and spleen, they were uncountable.

Taking the individual organs, the spleen showed the most striking differences (figs. 1 and 2). In the experimental group it was very large—up to three times as long as in the control group where only slight enlargement above the normal was seen. Intense engorgement was also present, in fact spontaneous rupture was the cause of death in an experimental animal of another series. Microscopically all structure was lost within the lesion and bacilli were present throughout it.

The liver showed the fibrosis in the controls and the necrosis in the experimental animals particularly well (figs. 3 and 4). The lungs showed localised septal lesions in the controls compared with confluent pneumonic lesions in the others (figs. 5 and 6).

Discussion

The differences shown in these experiments can be demonstrated whether the number of organisms inoculated is small or large, though the survival time of the controls is mainly dependent on the dose. This is in agreement with Kettle's observations (1927) on tuberculous lesions in the presence of a non-specific irritant such as turpentine.

It does not follow that all strains of tubercle bacilli will give identical lesions, those here described apply to five of the six strains so far tested, the remaining strain, though also from human sputum, was more virulent for

for three months, thereafter monthly to six months and again at nine months. As the milk inoculum produced enough opacity to interfere with the detection of growth a further subculture in glucose broth was made. At the end of six months the biochemical reactions of all viable strains were retested.

Results

Milk medium (a) *At 37° C* At the end of three months eight strains were living, the other four had died by the eleventh week. The clotting of the milk and the contraction and drying that had occurred rendered this set of tubes useless despite the paraffin seal and they were discarded.

(b) *At room temperature* The result was similar to that obtained with the cultures kept in the incubator, ten strains being viable after three months. This set was also discarded.

(c) *At 8° C* The majority of tubes showed no clotting and were thus more easily handled. At the end of three months nine strains were living, but only five survived for six months.

Broth medium (a) *At 37° C* So much evaporation occurred that the tubes were discarded after eleven weeks. Seven strains were viable at this time.

(b) *At room temperature* Although seven strains survived for three months, this set was also discarded because of the amount of evaporation that had occurred.

(c) *At 8° C* Unfortunately these cultures were contaminated by fungi at an early date.

Cooked meat medium. This proved to be the most satisfactory of the media tested. At three months all strains at each temperature were viable. After six months, nine strains maintained at 8° C were viable, eleven at room temperature and ten at 37° C. All these strains were tested for purity, colony form, morphology, fermentation reactions and acid production. Only minor changes were detected, mainly in the morphological and colonial characteristics.

At the end of nine months there were eight surviving strains at 37° C, eight at 8° C but only six at room temperature.

Discussion

Wadsworth (1927) suggested that *L. acidophilus* should be incubated at 35 - 36° C in a milk medium for 24 hours and stored at 4 - 8° C for 24 hours, whereas Rockefeller (1925) recovered lactobacilli from milk which had been sealed for 10 years. Again, White and Avery (1909-10) were able to maintain strains in milk for 31 days at 25° C, or at refrigerator temperature, without difficulty, whilst Enright, Friesell and Trescher (1932) maintained stock cultures in milk by bi-monthly transfers for over a year.

The results of our observations indicate that the maintenance of oral strains of *L. acidophilus* is not difficult. Milk medium, which is clotted by these organisms at 20° and 37° C, is less conveniently handled than cooked meat medium. Although the storage temperature does not seem to be of much importance, on general considerations it would seem preferable to keep the cultures at refrigerator temperature.

Summary

Twelve oral strains of lactobacilli were maintained without subculture for at least three months in cooked meat medium at 8°, 22° or 37° C.

give rise to freak staining as usual. The dyes principally used have been Grubler's granular methyl blue and the R A L brand (Kuhlmann, Paris) of eosin.

Apart from the preliminary mordanting, the method aims at following the directions given by Mann, and the proportions of the dyes in the mixture are those stated by him, namely, to 100 c c of distilled water add 35 c c of 1 per cent aqueous methyl blue and 45 c c of 1 per cent aqueous eosin. The proportions of these dyes are not always correctly given in reference books, and methylene blue cannot be substituted for methyl blue.

Ammonium molybdate was one of the myelin mordants used by Bolton (1897-98, 1898-99) for staining frozen sections of nervous tissues by the Weigert-Pal method. Its greatest use as a myelin mordant in my experience has been to revive sections of chromicised material, embedded in celloidin, which has turned green through prolonged keeping in dilute alcohol. Old material of this type is often wasted because the myelin stains very badly with the hæmatoxylin and the nuclei with stains such as alum carmine. Remordanting in ammonium molybdate restores to a large extent the affinity of these tissues for their respective dyes.

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The problems on which he was engaged during his sojourn in the Wellcome Laboratories were of a kind that demanded team work, and almost all he did was done in collaboration with others. For him this was no hardship. He had nothing of the recluse. He was fertile in ideas and active and critical in working them out, but he liked nothing better than discussing them with others, even in their embryo stages. His mind worked best in the open. The quantitative methods demanded in the assay of immunological reagents made a strong appeal to him, and in later years he often stressed the value of the discipline which such work imposed. He was equally attracted by the practical application of the reagents he helped to prepare and standardise in the prevention and treatment of disease. He never fell into the error of regarding the walls of the laboratory as the natural boundaries of medical science or of believing that the general principles that applied within them were inapplicable in the world outside. It appeared to him merely stupid to take great care to determine the effect of immunological reagents on experimental animals without taking equal care to measure their prophylactic or therapeutic effect in man.

His work during these ten years was concerned largely with problems relating to prophylaxis against diphtheria or scarlet fever. Most of his papers bear other names besides his own. R. A. O'Brien, P. Hartley, the late A. J. Eagleton, H. J. Parish, T. Dalling and others collaborated with him, or he with them. From 1924 to 1930 most of his work was done with Parish.

His contributions to the problem of Schick testing and diphtheria prophylaxis include papers on the Schick test and active immunisation (1923), on the preparation of stable toxin dilutions for Schick testing (1927-28), on the permanence of the Schick-negative state (1928) and on the reputed occurrence of diphtheria in Schick-negative reactors (1929). In 1924 he also published, in collaboration with A. J. Eagleton and R. A. O'Brien, a description of a method, based on Schick testing and immunisation, by which an outbreak of diphtheria in a school or similar institution could quickly be brought under control.

Between 1924 and 1932 he published eleven papers dealing with one problem or another in connection with the prophylaxis of scarlet fever or with the preparation and use of scarlatinal antitoxin. His papers with Parish on the standardisation of this reagent, published in 1927, formed, at that time, an important contribution to a difficult technical problem.

Other subjects coming within the essential activities of the laboratory and on which he published papers included the standardisation of tuberculin and of Shiga antitoxin. In relation to the former, he played a part in showing that the precipitin reaction does not afford a reliable measure of activity. But his

Lewis and Giant had shown, give rise to ulcerative endocarditis in a high proportion of congenitally defective heart valves. The second paper, published in 1936 and again in collaboration with S. D. Elliott, was of even greater practical importance. Making use of the methods of serological typing which had already proved so successful in tracing the source of infection in puerperal fever caused by hæmolytic streptococci, they investigated the occurrence of cross infections with these organisms in otorhinological wards. The result was a clear demonstration that such hospital infection was very frequent and often very serious. It became clear that the prevalent methods of nursing and isolation were failing to prevent the spread of infection from one patient to another and that new methods would have to be devised. Subsequent experience has shown that this is a general and not an isolated phenomenon, and the evidence that has accumulated since Okell and Elliott's paper was published has raised as an urgent problem the revision of our methods of controlling ward infections, not only in otorhinological wards and in fever hospitals but in general hospital practice as well.

These two investigations marked the end of Okell's active laboratory career. The rheumatoid arthritis which so severely crippled him during the last five years of his life began to cause serious trouble in the latter part of 1933, and a year later he was compelled to go into University College Hospital for treatment. He spent the summer of 1935 at Lewes without appreciable benefit, and in August he returned to hospital, there to remain for over a year. In the autumn of 1936 he went down to the Hospital for Officers at Brighton, remaining until October 1937. The active stage of the disease had at last become arrested, but he was left with an almost completely rigid spine and severe and permanent damage to many joints, especially of the lower limbs.

Okell's reaction to his painful, crippling and long-continued malady was something to marvel at. He took it not merely philosophically—fully prepared to make the best of a bad job—but in a spirit of optimism and even of light-heartedness which was an inspiration to all who knew him. Friends who called to cheer and comfort him were themselves consoled. It is extraordinary to think that the little skit entitled "On the quantitative study of tumours," which was published anonymously as an inset to the January 1937 number of the *Journal* and which brought delight to pathologists and others the world over, was written in the autumn of 1936 when his physical state was just about at its lowest. We must not fail to mention also his delightful, witty and amusing contributions to the *Lancet's* "Gains and scruples" series, entitled "From a bacteriological back-number." Published during January 1938, just a year before his death, they show better

problems of bacteriology and pathology as he felt they deserved, instead of cramping and distorting them within the limits set by an overcrowded hospital curriculum. In the last year of his life he was expanding this supervisory teaching. Had he lived, there can be little doubt that he would have taken an increasingly important place in the system of pre-clinical medical education that is peculiar to the older universities.

Okell's association with this *Journal* covered a period of five years. He was appointed an assistant editor in July 1932 and resigned on accepting office as editor of the *Journal of Hygiene* in July 1937. He took his duties very seriously and, there can be no doubt, thoroughly enjoyed the work. During the years of his invalidism it was obviously a source of deep satisfaction to him to feel that he was not wholly laid aside, but able to be of some use in the world. On 26th August 1935 he wrote — "Don't hesitate to send me stuff, while I can I like to do it, and there are not many other things one can do while lying on one's back." Even during the period of his greatest incapacity he continued to perform his editorial duties, his daughter acting as his amanuensis. In reading MSS he had a remarkable faculty for sifting the wheat from the chaff, and his wide knowledge of bacteriological literature stood him in good stead when he came to assess the degree of originality in papers submitted for publication. As an editorial colleague he was ideal, and his frequent letters nearly always contained much general entertaining matter in addition to what was strictly business.

Apart altogether from his scientific and professional work, Okell was a man whose lively mind evoked a response in all with whom he came in contact. Next to his interest in science came his interest in literature, and, as any serious student must, he specialised in both. His knowledge of the work of Marlowe and his contemporaries was of the same high order as his knowledge of bacteriology. But he was no rigid specialist. He knew more than a little of the literature of theology and ethics and was quite prepared to support an argument in either.* Art and music

* "I have just been reading with much interest Moffatt's modern translation of the New Testament. Much as I love the authorised version I have to admit that in many places the modern translation is streets ahead in getting the humanity out of the story. Much of the New Testament is intensely vivid and natural in its narrative while in the authorised version the mental imagery is superb but the simplicity is for complex reasons submerged. For example the story of Paul's shipwreck in the Acts reads in Moffatt like a page of Robinson Crusoe and the passages in the Gospels and the Acts where Christ or Paul as the case may be answers his accusers in the Roman Court are to my mind incomparably more graphic than in the old version. The same with the Pauline Epistles. Much of the incomparable grandeur of language is wanting, but the Epistles become perfectly straightforward bits of popular preaching instead of the complicated theological treatises of the old version. I believe that it would do much to revive the waning popular interest in the Bible if these modern versions were more freely circulated."

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of surprise and dismay Yet after reading the preface one realises that this little manual has been quite literally called for and that it is in effect a comprehensive answer to those who have sought or might seek the author's advice and assistance in this field of histological technique The title, moreover, gives a very modest indication of the real scope of the work Methods for the demonstration of all the more important structures and morbid products in the central nervous system are described and the worker in neuropathology who is not particularly concerned with tumours will nevertheless find much of value in its pages The inclusion of one or two really demonstrative methods for gha fibres would, in fact, have made the book a complete primer of neurological technique

"The notes are confined to a selection of methods which have proved satisfactory" Herein lies the chief virtue of the work Life is short and methods multiply, and details of one procedure that will work are of more value than the choice of a dozen that may not There are several fine plates which show that the methods described do work to great advantage but, as the author emphasises, good results can be obtained only by employing reagents of high quality, especially for fixation

In brief, this is an excellent practical manual, based on personal experience, clearly written and eminently helpful

Studies on the size of the red blood cells especially in some anæmias

By ERIK MOGENSEN Copenhagen Ejnar Munksgaard, London
Humphrey Milford, Oxford University Press 1938 Pp 216,
43 text figs 12s 6d

The technique elaborated by Price-Jones for investigation of the variations in diameter of the red cells has been employed by a number of research workers in hæmatology, the majority of these have been content to use it as an accessory investigation and have not always appreciated to the full the significance of their findings; others have found it a means of supporting their views in defiance of its mathematical implications Dr Erik Mogensen bases his book upon a large number of estimations by the Price-Jones method supported by other and more routine hæmatological investigations, and he has recorded his findings and drawn his conclusions with care and discrimination A certain amount of this work has been done before, that upon normal subjects, cases of pernicious anæmia and microcytic anæmia by Price-Jones himself, that on other anæmias by many workers who are quoted by Dr Mogensen The book owes its value to the author having put into it his own extensive investigations on most of the varieties of anæmia, and to his having compared them with the findings of others In so doing he has, perhaps inevitably, raised more problems than he has solved, a fact which makes his book especially stimulating to the research hæmatologist a considerable amount of work by others is confirmed and a certain amount debunked, whilst his own views are recorded with due moderation

In the first chapter the history of the measurement of the erythrocytes is recorded from the initial observations of van Leeuwenhoek, while the second deals with the Price-Jones technique and the ways by which the measurements can be turned to statistical use This chapter, a hard one for the non-mathematically-minded reader, besides discussing the methods of calculating mean diameter and coefficient of variation, describes the means by which the author decomposes his curves The

reference for the virus research worker. To others outside this field it can have little appeal and to all who essay the task it will prove heavy reading. There is little uniformity either of purpose or of presentation, and one lays down the work with less real understanding of what viruses are or do than after reading, for instance, Sir Patrick Laidlaw's Rede lectures of 1937, which occupy but a fraction of the space.

The introductory section of 110 pages by Professor Doerr is an attempt to give a comprehensive view of the virus field as a whole. Most of the information in it is repeated by subsequent contributors. Bacteriophage is included in several places in the work, apparently because we know even less about its real nature than is known about the nature of viruses. Dr Elford contributes a large section on the size of viruses and bacteriophages. This is a subject which the author has made peculiarly his own and the section contains, therefore, a great deal of practical information, some of it not hitherto published. The author is to be congratulated on a clear presentation of the subject and on the wealth of data, particularly ultra-filtration data, which is made available. It is a pity that the limitations of the techniques which he describes are not discussed more frankly. The virus sizes in many cases are given with tolerance limits, which can only be described as optimistic.

Dr M. Kaiser contributes a useful and nicely illustrated section on staining methods for viruses. Dr Hartinger's article on fluorescence microscopy is interesting in relation to the fluorescence microscope but not very communicative on the subject of viruses. The difficult subject of inclusion bodies is admirably treated by Dr G. M. Findlay, the bibliography to this section extending to no less than 400 references. In the section on cultivation Dr C. Hallauer deals exhaustively with the problems of virus growth in association with tissue explants and Dr F. M. Burnet describes the technique of cultivation on the chorio-allantoic membrane of the developing chick, a method which since its introduction in 1931 by Woodruff and Goodpasture has found a steadily increasing field of usefulness. A particularly instructive part of this article deals with the methods of titrating viruses on the chorio-allantois and with the interpretation of the results.

The concluding section to this volume is by Dr W. M. Stanley on the biochemistry and biophysics of viruses. The writer reveals an expert knowledge of both animal and plant virus fields. In dealing with the former there is naturally some overlapping with Dr Elford's article. It is interesting to note that Stanley has decided at last to withdraw his earlier criticisms of the evidence by Bawden and Pirie that the nucleic acid in the tobacco mosaic protein is indispensable to the activity of the virus. This author's concluding statement that "the evidence available at present is assuredly too meagre to justify the classification of the elementary bodies of vaccinia or, for that matter, of the tobacco mosaic virus protein with either ordinary molecules or ordinary organisms" will meet with the approval of most readers.

The most serious objection to this excellent book is its price—rather more than £5, 10s unbound. Even with the 25 per cent discount allowed to foreign purchasers, this figure must be very deterring. We are informed that the second half of the complete work, to appear shortly, will contain chapters on (a) virus agents in relation to infection, (b) natural and acquired immunity, and (c) the experimental investigation of plant viruses. Two appendices containing summaries of animal and plant viruses together with a subject index will complete the work.

bacteriology, not as a guide to technical procedures but as an outline of bacteriology as applied to clinical medicine

After some brief introductory remarks on apparatus, methods, and the classification of bacteria, a considerable amount of attention is given to infection and immunology in its many aspects, a useful chapter being included on the management of infectious diseases. Subsequent chapters deal with the infections associated with a particular organism or group of organisms, *e g* streptococcal, pneumococcal, coliform and enteric infections etc, virus infections and one on normal bacteriology. In the last-named the section on the alimentary canal is particularly useful. There is a short appendix on the collection of bacteriological specimens, routine staining methods, commonly used culture media, routine serological tests and the preparation of vaccines.

The book contains a disconcerting number of inconsistent statements (compare for example the differential reactions of the dysentery bacilli as given in the tables on pp 219 and 227), and minor inaccuracies, for example "Chas Hearsan & Co" (p vi), the "convex" mirror of the microscope (fig 2), "insulation" for inoculation (fig 20), "injest" for ingest (p 47), "objection" for objective of the microscope (p 244), "blood strain" for blood stream (p 245), "Ziehl-Neelson" (p 246), "Hampster" (p 248), "V metchinkovi" (p 318), "Mandelan" (p 359), the "shot-like pupules" of smallpox (p 362), "the 37 per cent incubator" (p 408) and so on. Jenner's classical inoculation experiment with cowpox to protect against smallpox is stated to have been made "in the middle of the nineteenth century". Both hurried production and careless revision are clearly indicated.

Most of the illustrations are quite good, a few are frankly poor and teach one nothing, *e g* the tubes of culture media.

As it stands one hesitates to recommend this book to students, with careful revision it could be rendered both reliable and useful.

Die Entstehung des Lebens durch stetige Schöpfung

By IGNAZ LICHTIG. Amsterdam. N V Noord-Hollandsche Uitgevers Mij. 1938. Pp xx and 371, 4 text figs. Dutch guilders 6 (bound 7 50).

It is curious that when confronted with a difficult synthesis and interpretation of fact, many scientists should prefer the fire to the frying pan. For instance, in order to explain the similarity between 5 per cent of the floras or faunas of two continents, authors have simply postulated a land bridge connecting them, without realising that they are then confronted with the greater difficulty of explaining why 95 per cent of the inhabitants of these two continents are different. The author of this book avoids this particular pitfall but falls into a much more serious one. The anomalies of discontinuous geographical distribution of related organisms and the difficulty of finding direct ancestors among fossils lead him to suppose that each species has been independently evolved from its own Protist ancestor. But this polyphyletic view of evolution provides no explanation of the facts of morphology, or of the intricate structural resemblances between organisms which we regard as related by genetic affinity and common descent. That man should have evolved is, from the physical point of view, a highly "improbable" event, so is the evolution of the chimpanzee, of the gorilla and of the orang. But that the extraordinary degree of resemblance between these species should be due to *independent*

for example, and much of the hæmatological chapter is theoretical, while many little snippets of information are provided which it would surely have been better to omit on the assumption that the readers of this book had undergone a sufficient medical and scientific training to be able to understand the subject matter. As an example (p 291) "Mitosis is indirect cell division or karyokinesis. It is the typical mode of division of active somatic cells and germ cells." If the author assumes, as he does, a knowledge of what is meant by a somatic cell and a germ cell, why not assume the same for mitosis? By dispensing with much of this irrelevant and unnecessary matter it ought to have been possible to produce a smaller, more convenient and less expensive volume than the present mighty tome.

Apart from these criticisms, we can only speak in very favourable terms of Dr Gradwohl's new edition. The general arrangement, printing and photography are excellent and the accuracy of the index and page references is beyond reproach. The method of arrangement of the different chapters is practical and helpful, usually commencing with methods of collecting and preserving specimens, followed by detailed descriptions of technical procedures and ending with the interpretation of the findings. Three hundred pages are devoted to hæmatology, as the author believes that the blood is one of the most important fields in laboratory work. Discussion of the origin of blood cells, their nomenclature and the physiology of the hæmopoietic organs and blood cells is followed by details of technical procedures, special emphasis being laid on the Schilling method. The coloured plates of blood and bone marrow smears are very successful. A serious omission, however, especially to workers in this country, is the failure even to mention the work of Price-Jones. We think it would have been well also to have included some more accurate method for the estimation of red cell fragility. The book concludes with a comprehensive chapter on parasitology and tropical medicine, in the revision of which the author has had the co-operation of Professor Pedro Kouri of the University of Havana, Cuba. The abundant references to recent literature should be useful.

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